

Lentiviral vectors for gene therapy of Gaucher disease

Katherine L. Aitchison

Institute of Child Health

University College London

A thesis submitted for the degree of Doctor of Philosophy

April 2015

Declaration

I, Katherine Aitchison, confirm that the work presented in this thesis is my own with the following exceptions. In chapter 6, the modification of bone marrow with the eGFP vector and subsequent transplantation and culling of mice was performed by Dr Maria Alonso-Ferrero and Dr Marlene Carmo (UCL Institute of Child Health), the culture of osteoclasts from *GBA*^{-/-} mice for Figure 5.5 was performed by Matthew Reed (Royal Free Hospital, Hampstead).

Acknowledgements

Firstly my heartfelt thanks go to my primary supervisor Steven Howe for his constant supervision, generous instruction and unwavering support. I must also thank my secondary supervisors Simon Waddington and Christine Kinnon for their valuable insights and contributions throughout the course of this project. I would also like to thank the Great Ormond Street Hospital Children's Charity for funding this project and for giving me the opportunity to be a part of it.

My deepest thanks to Ahad Rahim who provided the original vector that much of this work is based on and provided indispensable advice on countless occasions. I also owe a debt of gratitude to my collaborators Tim Arnett, Derralynn Hughes and Matthew Reed without whom chapter 6 of this thesis would not exist and who kept me sane whilst fighting to produce "even one osteoclast". Simon Heales and Derek Burke in the Enzyme lab at GOSH have also incurred my gratitude for their assistance in teaching me the 4MUG assay and for allowing me bench space whenever I asked for it.

Almost everyone in MIU has provided me support of one form or another over the course of my PhD, be it scientific, moral or, when I was in dire need, chocolatey, and I would like to thank them all. In no particular order: Emma, Celeste, Claudia, Marlene, Maria, Ben and Roman who could always be relied on to make the trip to the Rugby on a Friday night; Conrad; Albert, Luisa, Karolin and everyone else who has been a part of MIU over the years and who therefore deserves inclusion. Of course I also have to mention my fellow 2010 students and members of the thesis club – Dessi, Anna, Mustafa and Julien; we shared each other's highs and lows and made the PhD a truly collaborative experience.

My thanks are also owed to all of my friends outside of ICH who have navigated the tempestuous journey through the PhD with me and have been unfailingly supportive and cheerful even in the face of true pessimism. In particular my thanks go to Aleisha who was the best scientific proof-reader a girl could ask for as well as a wonderful friend.

Finally I have to thank my mum without whom none of this would have been possible; her belief in me and abiding love and support allow me to go places I never would have thought possible.

For Donna

“One more step along the world I go”

Abstract

Gaucher disease (GD), a recessive disorder characterised by hepatosplenomegaly, pancytopenia and skeletal complications, is caused by deficiency of the enzyme glucocerebrosidase (GC). GD leads to the accumulation of glucocerebrosides within macrophages, particularly in the liver and spleen. Current treatment is limited to enzyme replacement therapy (ERT) which is effective for most symptoms however skeletal problems are slow to respond. Treatment also has significant cost and impact on quality of life as infusions must be administered every two weeks. GD is a candidate for gene therapy as bone marrow transplantation has been shown to be curative which serves as a proof-of-concept that correction of haematopoietic stem cells (HSCs) can alleviate disease. This project produced lentiviral vectors carrying a range of constructs. GC was modified to contain a protein transduction domain (PTD) which could facilitate cross-correction of untreated cells *in vivo*. Recombinant vectors carrying PTD-GBA cDNA corrected the metabolic defect in patient-derived fibroblasts with levels of enzyme activity restored to within the healthy range. Transduced cells secreted active protein, uptake of which by untransduced cells was mediated by fusion of a PTD to the C- but not the N-terminus of the enzyme. The skeletal complications of GD are likely to be caused by enzyme deficiency in the osteoclast, a cell of haematopoietic origin. Therefore it is possible that by transducing HSCs we will be able to alleviate skeletal symptoms. To this end it is shown that modification of HSCs does not affect their ability to generate osteoclasts. It is also demonstrated that osteoclasts derived *in vitro* from the neuronopathic GD mouse model have increased activity and this could be a useful model for osteoclast correction when treating GD. In conclusion, this project generated lentiviral vectors for use in treating Gaucher disease. Further work should include correction of the osteoclast phenotype and further investigation of the potential for cross-correction *in vivo*.

Table of Contents

1	Introduction	15
1.1	Gaucher disease	15
1.1.1	Discovery	15
1.1.2	Lysosomal biology	18
1.1.3	Genetics	20
1.1.4	Pathophysiology	23
1.1.5	Bone disease	28
1.1.6	Current treatment	31
1.1.7	Animal models	37
1.2	Gene therapy	40
1.2.1	History	40
1.2.2	Clinical trials	42
1.2.3	Vectors	47
1.2.4	Lentiviruses	53
1.2.5	Protein transduction domains	63
1.3	Gene therapy for Gaucher disease	66
1.4	Summary and Aims	70
2	Materials and Methods	71
2.1	Materials	71
2.1.1	Cloning	71
2.1.2	Plasmid preparation	71
2.1.3	Virus production	71
2.1.4	Virus titration	72
2.1.5	Cell culture	72
2.1.6	Assays	72
2.1.7	Western blotting	73

2.1.8	Immunocytochemistry.....	73
2.1.9	Antibodies	74
2.1.10	Solutions	75
2.2	Methods.....	76
2.2.1	PCR amplification	76
2.2.2	Restriction digests.....	76
2.2.3	Klenow polymerase treatment (filling 5'-protruding ends).....	76
2.2.4	Ligation.....	76
2.2.5	TOPO cloning.....	77
2.2.6	Transformation	77
2.2.7	Gel electrophoresis	77
2.2.8	Plasmid preparation.....	77
2.2.9	Propagation of mammalian cell lines.....	78
2.2.10	Virus production	79
2.2.11	Virus titration	79
2.2.12	Osteoclast culture	81
2.2.13	Bone marrow cultures	82
2.2.14	Assays.....	83
2.2.15	Western blotting.....	84
2.2.16	Immunocytochemistry.....	85
2.2.17	Software.....	86
3	Vector production and characterisation.....	87
3.1	Aims	87
3.2	Introduction	87
3.3	Results.....	89
3.3.1	Patient interest in gene therapy for type I Gaucher disease	89
3.3.2	Vector production.....	91
3.3.3	Glucocerebrosidase activity can be determined with fluorometric assays	95

3.3.4	Vector constructs produce functional glucocerebrosidase protein	97
3.3.5	Enzyme activity levels peak at day 3 post transduction	100
3.3.6	Integrated vector can restore enzyme activity in patient fibroblasts	104
3.3.7	Chitotriosidase activity cannot be measured in fibroblasts <i>in vitro</i>	106
3.4	Summary	109
4	Secreted glucocerebrosidase for the cross-correction of untransduced cells	111
4.1	Aims	111
4.2	Introduction	111
4.3	Results.....	114
4.3.1	Developing assay for secreted protein	114
4.3.2	Cloning vectors.....	120
4.3.3	pHR-SFFV-GBA-WT TAT.....	120
4.3.4	The addition of the protein transduction domain does not affect expression or activity of GC	126
4.3.5	Transduced cells secrete GC protein via an endogenous secretion signal	128
4.3.6	Uptake of GC can be mediated by protein transduction domains	132
4.4	Summary	134
5	Investigating gene therapy as a potential treatment for the osteoclast.....	135
5.1	Aims	135
5.2	Introduction	135
5.3	Results.....	138
5.3.1	Comparison of osteoclast number in BL6 and CD1 mice.....	138
5.3.2	Osteoclasts can be derived from transduced bone marrow	140
5.3.3	Osteoclast numbers in healthy vs GD mice	145
5.3.4	Viral transduction of osteoclast cultures	147
5.3.5	CFU-assay in transduced bone marrow.....	149
5.4	Summary	151
6	Discussion	153

7	Appendix	168
7.1	Papers presented	168
7.2	Sequence alignment of <i>GBA</i> including mTAT or WT TAT fusion.....	169
7.3	Gene therapy factsheet sent to Gaucher patients	172
7.4	Results of survey to Gaucher patients	177
7.5	Free text answers to Q5.....	181
8	References	183

Table of Figures

Figure 1.1 Lysosomal storage disorders.....	17
Figure 1.2 Schematic showing the <i>GBA</i> gene, cDNA, mRNA and amino acid sequences...	22
Figure 1.3 Graph showing the number of results from a PubMed search for the term "gene therapy" plotted against year	41
Figure 1.4 The number of gene therapy trials reported by two sources from 1989-2013.	46
Figure 1.5 Structure of the Gag and Gag-Pol polyproteins of HIV-1.....	55
Figure 1.6 Development of first and second generation lentiviral vectors from the HIV-1 provirus	62
Figure 1.7 Cells of the haematopoietic lineage showing differentiation of the macrophage and osteoclast from the HSC.	69
Figure 3.1 Patient response to gene therapy for GD questionnaire.....	90
Figure 3.2 Removal of IRES- <i>eGFP</i> to give the pHR- <i>GBA</i> plasmid	93
Figure 3.3 Fluorescence assays for the detection of glucocerebrosidase activity.....	96
Figure 3.4 Glucocerebrosidase expression and activity in HEK293T cells	99
Figure 3.5 Glucocerebrosidase expression and viability over time in transduced cells. ..	102
Figure 3.6 Expression and activity of glucocerebrosidase is restored in patient fibroblasts after transduction with LNT- <i>GBA</i>	105
Figure 3.7 Chitotriosidase cannot be measured in cultured fibroblasts.	108
Figure 4.1 Comparison of the levels of autofluorescence produced by different preparations of FDGlu substrate in medium	115
Figure 4.2 Autofluorescence of fluorescein- β -D-glucopyranoside in media with and without conduritol- β -epoxide.....	116
Figure 4.3 Detection of velaglycerase alfa by enzymatic assay and western blot	118
Figure 4.4 Cloning strategies to generate fusion constructs	122
Figure 4.5 Schematic showing vectors produced	124
Figure 4.6 Addition of the mTAT PTD does not affect GC expression or activity	127
Figure 4.7 Secretion of GC from transduced cells	130
Figure 4.8 A 14-day time course showing GC activity within the supernatant of transduced cells reveals secretion of enzyme	131
Figure 4.9 Cross-correction of <i>GBA</i> ^{-/-} LCLs by protein secreted from transduced HEK293T cells	133
Figure 5.1 Surveyed type I patients find skeletal problems a significant aspect of living with Gaucher disease.....	137
Figure 5.2 <i>in vitro</i> osteoclast differentiation differs in mice of different strains	139
Figure 5.3 Osteoclasts can be derived from a genetically modified, autologous bone marrow transplant.....	142
Figure 5.4 <i>in vivo</i> osteoclasts from mice transplanted with eGFP transduced bone marrow.....	144
Figure 5.5 <i>in vitro</i> osteoclast generation in <i>GBA</i> ^{-/-} mice and healthy controls	146
Figure 5.6 <i>in vitro</i> transduction is most effective when performed at day 3 of osteoclastogenesis.....	148
Figure 5.7 Colony forming assay with transduced <i>GBA</i> ^{-/-} bone marrow	150

Table of Tables

Table 1.1 Subtypes of GD with associated symptoms	27
Table 1.2 Altered cytokine production by activated macrophages and the effect on osteoclasts/osteoblasts	30
Table 1.3 Viral types used as gene therapy vectors adapted from (256)	52

Abbreviations

4-MUG	4-methylumbelliferryl- β -D-glucopyranoside
BM	bone marrow
BSA	bovine serum albumin
CBE	conduritol- β -epoxide
ChT	chitotriosidase
CMV	cytomegalovirus
cPPT	central polypurine tract
DAPI	4'-6-diamidino-2-phenylindole
DMEM	Dulbecco's modified essential medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ECL	enhanced chemiluminescence
eGFP	enhanced green fluorescent protein
Env	Envelope protein
ERT	enzyme replacement therapy
FDGlu	fluorescein di- β -D-glucopyranoside
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
<i>GBA</i>	glucocerebrosidase gene
GC	glucocerebrosidase protein
GD	Gaucher disease
GDI	Gaucher disease type I
GlcCer	glucocerebroside
HEK293T	human embryonic kidney cell line
HIV-1	human immunodeficiency virus 1
HRP	horseradish peroxidase
HSC	haematopoietic stem cell

HSCT	haematopoietic stem cell transplant
IN	integrase
IRES	internal ribosome entry site
LB	Luria-Bertani
LCL	lymphoblastic cell line
LSD	lysosomal storage disorder
LT	Life Technologies
LTR	long terminal repeat
MA	matrix
M-CSF	macrophage colony stimulating factor
MEM	minimum essential medium
MGT	Marker Gene Technologies
MOI	multiplicity of infection
mTAT	modified trans-activator of transcription
NaT	sodium taurocholate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGE ₂	prostaglandin E2
SFFV	spleen focus-forming virus
PTD	protein transduction domain
PVDF	polyvinylidene fluoride
RANK-L	receptor activator of nuclear factor kappa-B ligand
RPMI	Roswell Park Memorial Institute medium
RRE	Rev response element
SRT	substrate reduction therapy
TAT	trans-activator of transcription
WPRE	woodchuck hepatitis virus posttranscriptional regulatory element

1 Introduction

1.1 Gaucher disease

1.1.1 Discovery

Gaucher disease (GD) is one of the most prevalent lysosomal storage disorders; a family of syndromes which cause accumulation of metabolic substrates within the lysosome of cells. It was first described in 1882 by a French medical student who reported a female patient with a greatly enlarged spleen which he thought to be cancerous. At post-mortem it was discovered that the spleen contained a large number of enlarged cells with a distinctive appearance (1). Subsequent to the publication of this report, further patients were identified and the swollen cells, identified as macrophages, were found to be symptomatic of GD and so came to be known as 'Gaucher cells' (2).

Further investigations revealed GD to be caused by a deficiency of the enzyme glucocerebrosidase (GC) (3) the gene for which localises to chromosome 1q21 (4). It is now known that GD is an autosomal recessive disorder affecting approximately 1 in 100,000 births in Europe (5) although this figure is significantly higher in the Ashkenazi Jewish population which has a carrier frequency of approximately 1 in 17 (6).

Gaucher disease is one syndrome in a family of over 50 metabolic disorders known as lysosomal storage disorders (LSDs). It is thought that, collectively, LSDs affect around 1 in 7,000 live births although it is difficult to accurately assess prevalence due to a high number of asymptomatic individuals and a lack of coherence in the literature (7).

Metabolism is an incredibly intricate process performed by a network of enzymes which sequentially degrade a macromolecule into its component parts. This is an important process as the released monomers are then available for use elsewhere in the cell or are

made available throughout the body. Mutations in any one of the metabolic enzymes can lead to accumulation of a substrate and when this accumulation occurs in the lysosome, the syndrome is classified as an LSD.

Figure 1.1 shows part of the lipid metabolism pathway which occurs within the lysosome; the key enzymes are labelled, along with their associated LSD. Although this work is concerned primarily with the development of gene therapy for the treatment of GD, the same principles could be used to treat other LSDs because, in many cases, storage is seen primarily in cells of the haematopoietic system, as in GD (8).

Despite being closely related in aetiology, LSDs tend to be clinically distinct and share only a limited number of pathological features. Although not all of these are associated with every LSD, common symptoms include: hepatosplenomegaly (swelling of the liver and spleen), dysmorphic facial features, cardiac complications, neurological involvement and skeletal abnormalities. The severity of any individual LSD is also hard to estimate because disease exists on a spectrum, from patients who are so mildly affected that they go undiagnosed, to levels which are incompatible with life (9, 10).

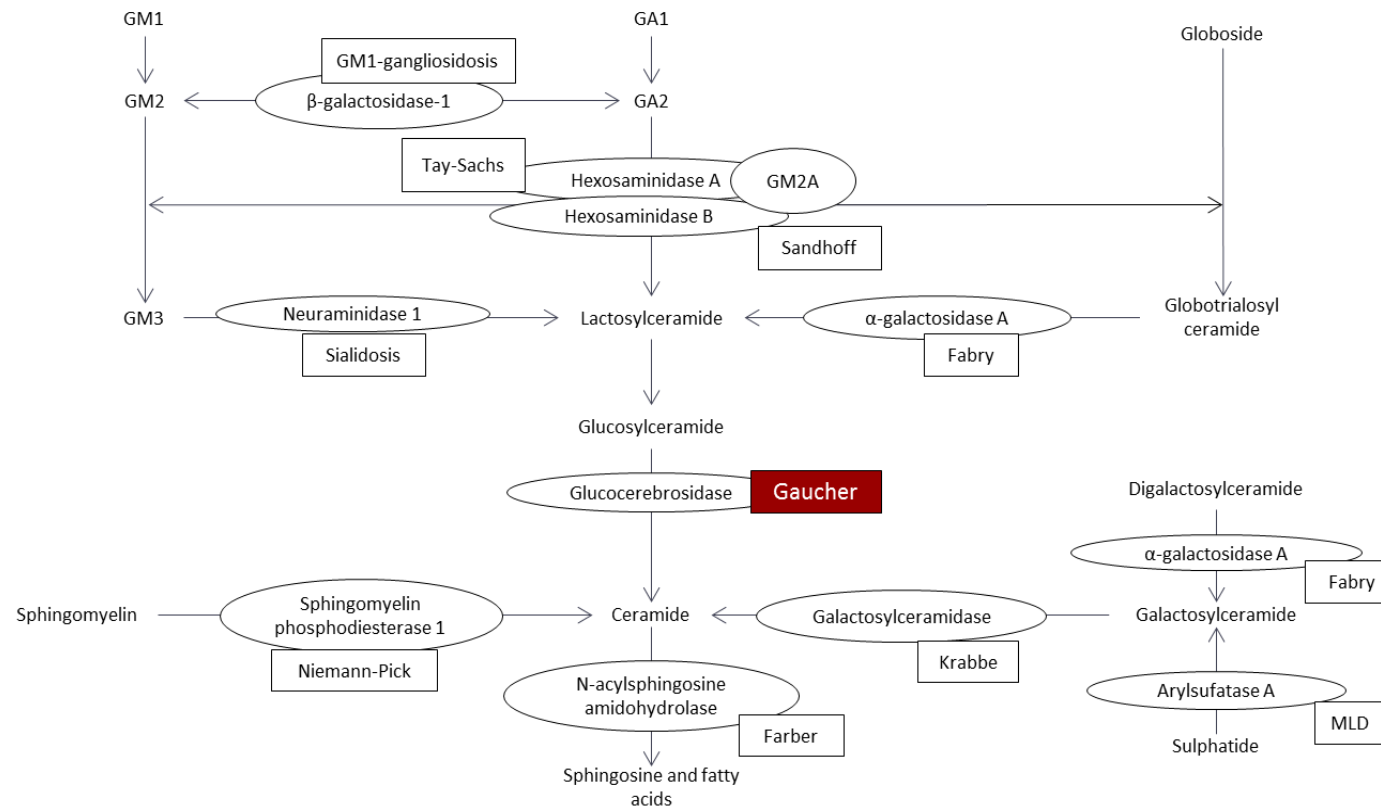


Figure 1.1 Lysosomal storage disorders

Metabolism of ingested lipids in the lysosome is performed by a network of enzymes (rounded boxes) acting sequentially to degrade molecules into products for reuse within the body. Mutations which prevent the activity of any enzyme in the network can severely affect the activity of the affected cell leading to disease (square boxes). GA: ganglioside, GM: monosialic ganglioside, GM2A: GM2 ganglioside activator, MLD: metachromatic leukodystrophy.

The characteristic feature shared by all LSDs is the accumulation of a substrate within the lysosome of cells which allows for classification based on the substrate accumulated (8):

- Mucopolysaccharidoses (I-VII)
- Sphingolipidoses
- Oligosaccharidoses
- Glycogen storage disease type II (Pompe disease)
- Lipidoses

There are additional classes of LSD which include disease caused by non-enzymatic defects but the categories above contain the most common disorders (8).

GD is classified as a sphingolipidosis because affected cells accumulate glucocerebrosides (GlcCer) which are a class of lipid with a backbone of sphingoid bases and a glucose head. In unaffected individuals the glucose head is removed from glucocerebrosides in a reaction catalysed by glucocerebrosidase (11). This allows for recycling of the glucose and further digestion of the sphingolipid to provide monomers that can re-enter the sphingolipid biosynthesis pathway (11, 12). In GD, patients have genetic mutations which result in the absence of functional GC leading to accumulation of glucocerebrosides within the lysosome (4, 13, 14).

1.1.2 Lysosomal biology

The lysosome is the final compartment of the endocytic pathway and is responsible for the degradation of macromolecules resulting from endocytosis or the autophagocytosis of subcellular components. Lysosomes were first identified by Christian de Duve in 1955 while he was investigating the subcellular location of glucose-6-phosphatase by differential centrifugation of cellular compartments. He discovered a membrane-bound

organelle, characterised by the presence of enzymes with a lytic action (15, 16), which he named the lysosome and which won him the 1974 Nobel Prize in Medicine or Physiology.

Within a cell there are two routes for degrading proteins: the ubiquitin-proteasome system which is mostly involved in the processing of small, short-lived cellular proteins; and the endosomal-lysosomal network. The lysosome receives molecules being trafficked into the cell as well as intracellular moieties intended for degradation, which first pass through the autophagosome (17, 18). Although it was first identified as containing the enzyme acid phosphatase, it is now known that the lysosome contains over 60 hydrolytic enzymes which combine to digest macromolecules present within the cell (19).

Lysosomal hydrolases are manufactured in the endoplasmic reticulum and transported through the Golgi network where the majority acquire a mannose-6-phosphate (M6P) group, which enables sorting to pre-lysosomal compartments through the M6P receptor (20, 21).

Although this is the usual route of delivery to the lysosome, there are other methods of transport. In I-cell disease (mucopolipidosis type II) patients are deficient in the enzyme N-acetylglucosaminyl phosphotransferase meaning that the formation of the M6P signal does not occur. This leads to the majority of hydrolases being secreted rather than delivered to the lysosome. However, some enzymes, including GC, still reach their intended cellular location (22). This is because GC does not acquire an M6P tag during synthesis and instead binds to lysosomal integral membrane protein type 2 (LIMP2) in the endoplasmic reticulum. LIMP2 is responsible for the trafficking of GC through the Golgi network and into the lysosome through a direct association which occurs in the endoplasmic reticulum (23, 24). The association between GC and LIMP-2 is maintained through the Golgi body where exit and entrance to the lysosome is co-ordinated by two

phosphatidylinositol 4-kinases (25). Dissociation of the two proteins in the lysosome occurs as a result of the low luminal pH.

1.1.3 Genetics

The gene for glucocerebrosidase (*GBA*) was cloned and mapped to chromosome 1q21 in the mid-1980s (4, 26). Further investigation showed the gene to span 7.8kb and to consist of 11 exons (Figure 1.2) (27). Interestingly there is also a *GBA* pseudogene located downstream of the functional copy which appears to have arisen as a result of a tandem duplication but which has large deletions from exons two, four, six, seven and nine (27).

Synthesis of GC can be initiated from one of two ATG codons within the sequence. Transcription seems to occur predominantly from the upstream initiator codon although deletion of either ATG does not affect the overall expression level *in vitro*. The initiator codon used for transcription alters the length of the leader sequence at the N-terminus of the protein which is 39 or 19 amino acids long depending on the codon used. However this sequence is cleaved from the mature enzyme and therefore it is unlikely that there is any effect on enzyme activity (28). It has also been shown that there are two promoters which can be used to initiate *GBA* transcription. The second, upstream promoter has an associated CpG island which is a common feature of housekeeping genes and may explain the ubiquitous expression of GC (29).

Translation of the *GBA* gene yields a 60kDa protein which has a modular structure consisting of three domains which were identified when the X-ray structure of the enzyme was published in 2003 (30). The catalytic activity of the protein is associated with domain III which is formed from amino acid residues 76-381 and 416-430.

The first pathogenic mutations in *GBA* (L444P (13) and N370S (14)) were described shortly after the gene was identified. To date, over 200 different mutations have been

reported, but the N370S and L444P mutations are the most prevalent (31). Certain populations have a higher than average incidence of a particular subclass of GD (for example, type I in Ashkenazi Jews (6) and type III in the Norrbotten region of Sweden (32)), but even when alleles are considered independently of patient origin, N370S and L444P are the most commonly found mutations (31, 33). Mutations reported to date include: 203 missense, 18 nonsense, 36 indels, 14 splice junction variants, and 13 complex alleles (containing multiple mutations), in addition to recombination events (31).

Many of the mutations described in *GBA*, including N370S, have structural effects on the protein and disrupt the protein folding which leads to retention in the endoplasmic reticulum and degradation of a catalytically active protein (34, 35). The N370S mutation occurs in the active site of the enzyme but in a loop outside the catalytic domain causing a structural change to the protein which makes it less flexible (36). The L444P mutation also causes a structural change, this time within the hydrophobic core of the protein (30) and which appears to affect the interaction of the protein with the activator protein saposin C (37). Another common mutation is the insertion of a second guanine base at residue 84 giving the 84GG allele which results in premature termination of transcription and is predicted to severely compromise the enzyme as it has never been seen in homozygosity (33, 38). The locations of the three mutations are shown in Figure 1.2.

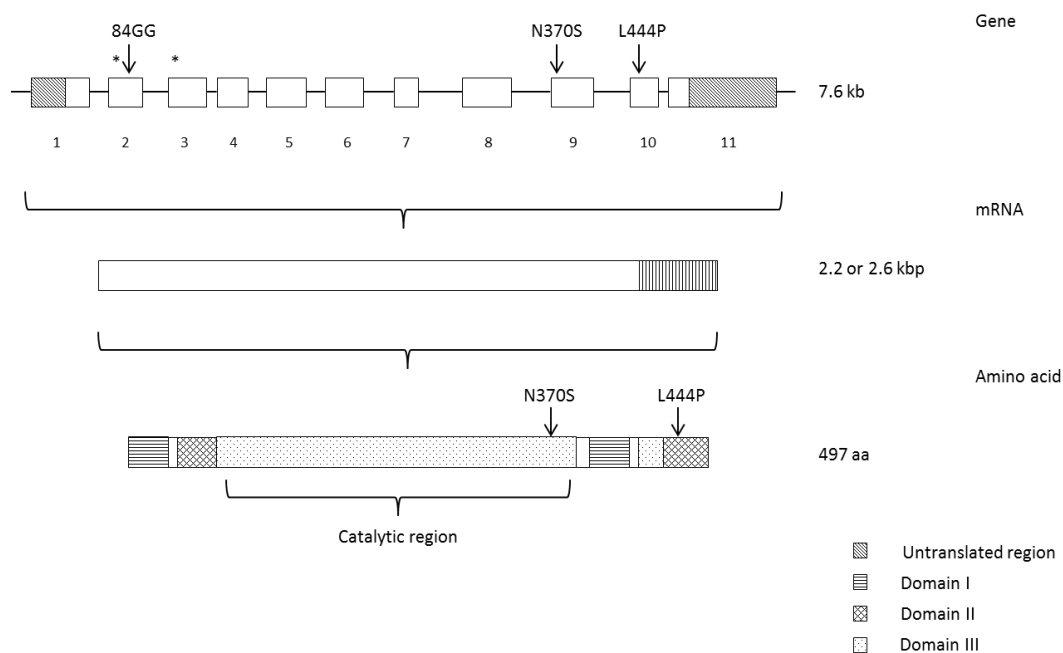


Figure 1.2 Schematic showing the *GBA* gene, cDNA, mRNA and amino acid sequences

Although many mutations have been described and *GBA* is well characterised, there is still only a limited understanding of genotype-phenotype correlation and the same mutations can be implicated across a spectrum of disease (39). There is even discordance found within twin studies (40, 41). One of the few examples of correlation that has been found is the N370S mutation (c.1226A>G) with non-neuronopathic or type I disease (42). However, even N370S homozygotes display a wide range of symptoms from asymptomatic to severe organomegaly with bone involvement (42, 43).

Similarly, the L444P (c.1448C>T) allele, which usually indicates neuronopathic disease, has been shown to exist on a continuum of symptoms ranging from moderate neurological and systemic symptoms to seizures and severe developmental delay (44).

Some studies have reported data which points to a dosage effect of individual alleles which results in the spectrum of disease seen in patients. For example; homozygosity for

the N370S allele results in milder symptoms and later onset than in heterozygous patients (33, 42).

A degree of phenotypic variability may be due to the effect of other genes which act as modifiers. Candidates include: *SCARB2* (the gene encoding the LIMP2 protein (45)), *CLN8* (46) and *PSAP* (47). *PSAP* encodes a polyprotein called prosaposin which is cleaved into four distinct saposins. Of these saposin C is a known activator of GC and mutations have been shown to cause GD (46). The *CLN8* gene was identified as a potential modifier of GD by a genome-wide screen and has been shown to be resident in the endoplasmic reticulum and to have a stimulatory effect on glucosylceramide synthase (48).

1.1.4 Pathophysiology

The symptoms of Gaucher disease are complex as it is a multisystem disorder which can cause significant haematological (49), skeletal (50) and neurological complications (51). The exact symptoms that a patient displays can be used to classify their disease based primarily on the presence or absence of neurological involvement, but also the age at which symptoms present (summarised in Table 1.1) (52).

The distinctive, swollen cells which were found in the spleen of the original GD patient are today known as Gaucher cells and are considered to be a hallmark of GD (2); the presence of these cells in a bone marrow biopsy is one of the main indicators used for diagnosis (53). Gaucher cells are macrophages which have a 'crumpled tissue paper' appearance due to the accumulation of the substrate, glucocerebroside (GlcCer), within the lysosome (11). GlcCer is released from the breakdown of cell membranes and therefore phagocytic cells such as the macrophage and the Kupffer cells of the liver are particularly affected and store large quantities of the substrate (54).

The mildest form of GD is type I in which patients predominantly experience visceral pathology including hepatosplenomegaly (with a spleen size of up to 20 times that of unaffected individuals (55)), anaemia and skeletal problems. This form is the most common and can be diagnosed at any age but is most commonly identified in adulthood. Symptoms can be effectively managed with treatment and patients have a normal life expectancy (53, 56).

Until recently, type I GD was defined as non-neuronopathic as patients did not display any neurological impairment. However, within the last decade a link has been established between type I GD and parkinsonism (56, 57). There have been anecdotal reports of Gaucher patients developing parkinsonian symptoms as far back as 1942 ((58) reviewed in (59)) but it was not until recently that a definitive link has been shown between type I GD and the development of early onset parkinsonism (59, 60). Subsequently it has been found that there is a high prevalence of GBA mutations amongst non-GD Parkinson's disease patients, making heterozygosity for GD mutations a risk factor for parkinsonism (61). The mechanism behind this association is still under investigation, but it has been suggested that it may be due to impaired mitochondrial function (62) or accumulation of mutant GC in Lewy bodies (63). However, parkinsonism in GD patients does not appear to be due to elevated levels of α -synuclein, which is associated with classical Parkinson's disease (64).

GD in patients with neurological disease can be classified as either type II or type III. Of these, type II is the most severe with symptoms arising in the first six months of life and deteriorating very rapidly leading to death at around 2 years of age (51). Type III is a milder form with less severe neurological symptoms and a later age of onset. The condition deteriorates over time but at a slower rate than in type II and is usually fatal in early adulthood (65).

The molecular basis for neurological disease in GD remains unclear, although degeneration appears to be due to neuronal loss and astrogliosis (66, 67). This may be caused by the accumulation of GlcCer within neurones (68) or by impaired autophagocytosis (69).

In addition to the three classical types of GD (I, II, III), further subtypes have been described in efforts to more accurately discriminate between groups of patients.

A subtype of type II which is often viewed as a distinct class of disease is named perinatal-lethal GD because affected individuals usually die within hours of birth, if not *in utero*. Symptoms indicative of this class of GD include hydrops fetalis, congenital ichthyosis, hepatosplenomegaly and arthrogryposis in combination with very low GC activity (<7% normal value) (70).

The delineation of type III GD has been subject to significant debate with three subclasses being suggested based on the severity of neurological symptoms (65). According to these subdivisions type IIIa patients would have milder visceral symptoms but early onset of neurological disease with seizures and horizontal supranuclear gaze palsy. In comparison, type IIIb is associated with significant hepatosplenomegaly as well as kyphoscoliosis and a barrelled chest, but later appearance of seizures. Although type IIIb is generally considered to be the class of GD seen at high frequency in the Norrbotten region of Sweden (known as the Norrbottnian type), the distinction between IIIa and IIIb is hard to make clinically and therefore these subdivisions are rarely used (71).

A subclass of type III GD used more frequently is type IIIc which is associated with calcification of the cardiac valves (atrial and mitral) and corneal opacity. Other visceral and neurological symptoms appear to be mild in these patients other than oculomotor

apraxia. Unusually for GD, type IIIc is very clearly associated with a specific mutation – the D409H substitution which strongly correlates with valvular calcification (72, 73).

Classification	Age of onset	Life expectancy	Neurological symptoms	Visceral symptoms
Type I (non-neuronopathic) ^(53, 56, 74)	Any	Relatively unaffected	None (Parkinsonism)	Hepatosplenomegaly, skeletal involvement (bone crises, osteonecrosis, osteomyelitis), anaemia, thrombocytopenia
Type II (acute neuronopathic) ⁽⁵¹⁾	<9 months	1-2 years	Strabismus, oculomotor apraxia, extrapyramidal symptoms, seizures, neck hyperextension, apnoea hypertonia	Pulmonary involvement, idiopathic fever, haemorrhage, splenomegaly
Type III (chronic neuronopathic) ⁽⁶⁵⁾	Childhood	Childhood	Oculomotor apraxia, myoclonic epilepsy, dementia, ataxia, spasticity	Hepatosplenomegaly, thrombocytopenia, anaemia, skeletal involvement, pulmonary involvement, oesophageal varices
Type IIIc (73, 75, 76)	0-20 years	20 years	Oculomotor apraxia	Mitral/aortic valve calcification, corneal opacity
Perinatal lethal ^(51, 70)	Birth/ <i>in utero</i>	<3 months	Hypokinesia	Non-immune hydrops fetalis congenital ichthyosis, facial dysmorphia, splenomegaly

Table 1.1 Subtypes of GD with associated symptoms

1.1.5 Bone disease

For type I GD patients, and to some extent type III, a significant pathology is bone disease which occurs in approximately 75% of patients (77).

Clinically skeletal involvement can include: restricted growth, osteopenia, osteonecrosis, lytic bone lesions and a specific class of bone malformation known as Erlenmeyer flask deformities (50, 78). Many patients also experience bone crises – periods of severe bone pain which can be very disabling. These crises, as with all GD symptoms, differ in frequency and intensity from patient to patient. The primary manifestations of bone disease are often a starting point for the development of secondary problems such as osteomyelitis, fractures, and osteoarthritis which cause further disability to the patient (50, 79).

The primary reason for the development of skeletal complications appears to be the infiltration of Gaucher cells into the bone marrow. This leads to an expansion of red marrow which alters the vascularity of the bone marrow compartment and allows the occurrence of infarction and thrombosis (50).

While infarction and thrombosis are both serious complications of GD, bone maintenance is a delicate balance between the two types of osteological cell – the osteoclast and the osteoblast – and so it is likely that there is also a cellular explanation for bone disease (80). Osteoblasts are mesenchymal cells which are involved in the formation of bone by laying down the matrix and differentiating into osteocytes (reviewed in (81)). Conversely the osteoclast, a haematological cell, is responsible for degrading and remodelling bone by breaking down the matrix (82). Both cell types can influence the behaviour of the other (83) and it is the balance between the activity of the two which ultimately results in a healthy skeleton (84).

As with all cells, the functions of osteoclasts and osteoblasts are influenced by cytokines, and so it is likely that altered cytokine production by macrophages within the bone marrow which have been activated by the accumulation of GlcCer has a role in bone disease ((85), summarised in Table 1.2).

The exact impact of GD on the cells of the skeletal system remains unclear although recently evidence has emerged that there is increased stimulus leading to the differentiation of osteoclasts in the GD setting (80, 86, 87). Evidence for the involvement of osteoblasts is less promising with a recent *in vitro* model showing that inhibiting GC had no effect on osteoblast activity (88), while another paper showed that there may be an uncoupling of osteoblast-osteoclast interactions in GD (86) and a recent *GBA*^{-/-} mouse model suggested that the osteoblasts was the most severely affected bone cell (89). Taken together, these studies suggest that a treatment strategy which directly targets the osteoclast may prove beneficial for the skeletal complications of GD.

Cytokine	Change in GD	Effect on osteoclasts	Effect on osteoblasts
Interleukin-1 β ⁽⁹⁰⁾	Increased	Stimulatory	-
Interleukin-6 ^(90, 91)	Increased	Stimulatory	-
Interleukin-8 ^(85, 92, 93)	Increased	Stimulatory	-
Interleukin-10 ⁽⁹¹⁾	Increased	-	Inhibitory
Macrophage-colony stimulating factor ⁽⁸⁵⁾	Increased	Stimulatory	-
Tumour necrosis factor- alpha ⁽⁹⁴⁾	Increased	Stimulatory	-
Prostaglandin E2 ⁽⁹³⁾	Increased	Stimulatory	-

Table 1.2 Altered cytokine production by activated macrophages and the effect on osteoclasts/osteoblasts

1.1.6 Current treatment

Originally, treatment for Gaucher disease was on a purely symptomatic basis. The most common measures were blood transfusions to treat anaemia and other cytopenias, with splenectomy when splenomegaly became too pronounced (95). However, splenectomy leaves patients at an increased risk of bacterial infections and is no longer used except when patients who fail to respond to treatment exhibit pronounced thrombocytopenia (96, 97).

Hematopoietic stem cell transplant (HSCT) has also been used for patients with severe disease and successful engraftment has been found to improve blood counts and reduce hepatomegaly in addition to correcting the underlying metabolic defect (98). It has also been shown that in some cases HSCT can stabilise bone disease (99, 100) and neurological condition in some type III patients (71, 101). Despite promising evidence of a curative effect, the mortality rate among HSCT recipients is around 10-25% and it is therefore not used as a routine treatment option (98, 102). However as HSCT has never been used with any degree of regularity and the majority of data is from the 20th century it is difficult to assess the true potential of the treatment. Given recent advances in technology, such as high-resolution human leukocyte antigen typing allowing for more accurate donor-recipient matching (103, 104) and improved stem cell mobilisation and collection regimens (105), some groups are now calling for re-evaluation of HSCT as a treatment option (106, 107).

1.1.6.1 Enzyme Replacement Therapy

The possibility of treating lysosomal disorders with replacement therapy was first suggested 50 years ago when it was observed that any substance taken up by endocytosis was likely to end up in the lysosome (15) (reviewed in (108)) but it was not until 1991 that this possibility was realised for GD (109).

Once the enzyme deficient in GD was identified (11), and a method for large-scale purification of the enzyme from human placenta was established (110, 111); researchers began experiments treating patients with infusions of enzyme. The first three studies used either unaltered enzyme ((112) reviewed in (113)), or enzyme encapsulated in either red blood cell membrane (114) or liposomes (115), but none were particularly successful in ameliorating disease.

An explanation for this failure came from a study performed in rats which showed that native GC enzyme administered intravenously localises predominantly to hepatocytes; cells which do not store GlcCer (116) and is therefore unlikely to alleviate disease. Rapid uptake of lysosomal enzymes by hepatocytes is a mechanism that has been seen in a number of studies (117-119) suggesting it to be a common mechanism of uptake for lysosomal proteins. Further investigations of the placental enzyme revealed it to contain a high percentage of galactose-terminated oligosaccharide side chains which interact strongly with a lectin expressed on hepatocyte cell membranes (120). The discovery that macrophages bear a receptor (macrophage mannose receptor; MMR) which interacts with mannose-terminal side chains provided a mechanism for targeting purified GC to its site of action (119, 121). The side chains of GC can be modified to target the enzyme to macrophages by the sequential removal of monosaccharide residues using the enzymes neuraminidase, β -galactosidase and β -N-acetylglucosaminidase (109, 121).

The first study of mannose-terminated placental glucocerebrosidase used a dose of 60U/kg administered once a fortnight which reduced organomegaly and improved haematological parameters with no serious adverse events (109). On the back of this trial the preparation was approved by the Food and Drug Administration (122) and was marketed by Genzyme as alglucerase (Ceredase®) but at considerable cost and with incredibly limited supply. In 1994 a second form of ERT, imiglucerase (Cerezyme®),

Genzyme), a recombinant form of protein produced in Chinese hamster ovary cells, was approved and became the primary treatment for GD (123, 124).

Since the approval of imiglucerase two other forms of ERT have been developed: velaglucerase alfa (VPRIV®, Shire Pharmaceuticals), a gene activated form of GC produced in human fibroblast cells (125, 126); and taliglucerase alfa (Elelyso®, Protalix/Pfizer) (127). Velaglucerase alfa is known as 'gene activated' as it is generated by the targeted recombination of the endogenous *GBA* gene with a promoter that would produce high levels of expression within fibroblasts (128). Adding kifunesine, a mannosidase I inhibitor, to the culture medium results in a protein with a high percentage of mannose-terminated side chains without the need for *in vitro* processing (129, 130). Taliglucerase alfa is produced in carrot cells and in 2012 became the first drug made in plant cells to win FDA approval (131, 132). Producing the enzyme in plant cells eliminates the need for post-translational modification to expose the mannose residues as more than 90% of the native side chains are mannose terminated (133).

A nine month randomised control study comparing imiglucerase and velaglucerase alfa in treatment naïve patients found no difference in the effectiveness of the two drugs, although there was a slightly increased risk of adverse events when treating with velaglucerase alfa (134). In addition, it has been shown that patients who switched from imiglucerase to velaglucerase alfa had no significant decline in the main GD parameters and that the change was well tolerated (135-137). Taliglucerase alfa has only been available to patients for a short space of time so there is less clinical data available but initial reports are promising with reduction of organomegaly and improvement in haematological parameters (127) and increase of bone marrow fat fraction (138) and stability in patients switching from imiglucerase (131). The results of a phase III clinical trial assessing the efficiency and safety of taliglucerase alfa in adult and paediatric

patients previously treated with imiglucerase showed the change to be well tolerated with maintenance of haemoglobin and platelet concentration as well as liver and spleen volume (139). Taliglucerase alfa was approved by the FDA for use in paediatric patients in August 2014 and importantly for many patients, is the only one of the four drugs available to be deemed kosher by the US Orthodox Union (140, 141).

The approval of these two variants of ERT was driven in part by viral contamination (Vesivirus 2117) at the Genzyme manufacturing plant in 2009 which caused a worldwide shortage and left many patients without treatment (142, 143). In addition to pushing velaglucerase alfa through the regulation process, a second effect of the Cerezyme shortage has been beneficial for this project as it has left patients less willing to rely on a treatment where supply could be interrupted at any time and, therefore, potentially more willing to consider trying genetic therapies and permanent correction to disease (Tanya Collin-Histead: personal communication).

It is also worth noting that ERT is an expensive treatment option costing around £100,000 per patient, per year in the UK (144, 145) and it is not curative meaning that any interruption to supply can lead to the recurrence of symptoms (146, 147). While the cost is not a major problem for patients in the UK, those in less developed countries such as India (148) and Pakistan (149) may have limited or no access to ERT because the cost is too high (146, 150). Some of these patients are supported by compassionate access programs such as those run by Genzyme (Massachusetts, USA) and the European Gaucher Association but such schemes are limited and a one-time, curative treatment may represent a more accessible option for these patients.

1.1.6.2 Response of bone disease to treatment

While ERT has been shown to be effective in treating the majority of visceral symptoms, evidence for the treatment of skeletal symptoms is less easy to interpret. In general, the

frequency and intensity of bone crises appears to decrease rapidly in response to ERT (151, 152) but some patients continue to experience crises and in some cases osteonecrosis can continue to develop asymptotically (153). Another measure of skeletal disease in GD is bone mineral density (BMD) which is highest among adolescents and then gradually declines with age (154). In keeping with this pattern, an analysis of patient data from the International Collaborative Gaucher Group registry showed that osteopenia was evident in children as young as five years and most pronounced in adolescents (155). It is therefore unsurprising that younger patients respond to ERT better with the most significant increase in BMD and reduction in both bone pain and bone crises (155, 156). Rosenthal *et al.* (1995) showed that bone density takes an average of 3.5 years treatment with ERT to return to normal values. The same study also showed that it takes a similar length of time for the marrow fat fraction to normalise in treated patients (157).

1.1.6.3 Substrate Reduction Therapy

Around 15% of patients display hypersensitivity to alglucerase/imiglucerase (158) and although this can be managed, there is another option – substrate reduction therapy (SRT). Patients are treated with an inhibitor of glucosylceramide synthase (miglustat, marketed as Zavesca®, Actelion) which limits the formation of GlcCer and has been shown to be effective in treating GD (159). The active ingredient in miglustat is *N*-butyldeoxynojirimycin (NB-DNJ), an iminosugar which was originally identified as an inhibitor of HIV infectivity (160, 161). Subsequent investigation showed it to be an analogue of D-glucose and a competitive inhibitor of glucosylceramide synthase which is the first enzyme in the glycolipid synthesis pathway (159). SRT has been shown to be effective in treating GD, particularly in reducing organomegaly (162) and with promising results emerging for the treatment of bone disease (163). However, utility is limited due to gastrointestinal disturbances including osmotic diarrhoea and weight loss (164). These

side effects are caused by the inhibition of intestinal disaccharidase enzymes by NB-DNJ which affects the digestion of carbohydrates (164). Although they can be controlled by dietary alterations, miglustat is a less preferable treatment option and therefore SRT is only offered to patients who do not respond to, or who display sensitivity to ERT (162).

1.1.6.4 Pharmacological chaperone therapy

An emerging treatment area for GD is the use of chaperone proteins which can be employed to augment the activity of mutant protein. Pharmacological chaperone therapy (PCT) is a potential treatment strategy as many mutations which cause GD are missense mutations which either prevent the protein folding into the correct conformation or which prevent its trafficking to the lysosome.

PCT was first hypothesised after the observation that β -galactosidase inhibitors could in fact restore enzymatic activity in fibroblasts derived from patients with G_{M1}-gangliosidosis (β -galactosidase deficiency, Figure 1.1) (165, 166). Enzyme inhibitors have also been shown to act as chaperones in Fabry disease (167), Pompe disease (168, 169) and G_{M2}-gangliosidosis (170, 171). It has taken many years to elucidate the mechanism of PCT but it appears that chaperones can stabilise the substrate-enzyme complex thereby enable the misfolded protein to be targeted to the lysosome (167, 171, 172).

Two compounds have been identified which act as chaperones for mutant GC in cells – isofagomine (IFG) (173, 174) and ambroxol (ABX) (175). Incubation of GD-derived fibroblasts with either compound results in increased levels of enzyme activity, lysosomal localisation of the enzyme and clearance of stored GlcCer.

A pilot study which treated 12 GD type I patients with ABX over a 6 month period showed the drug to be safe for use in people with GD (it has already been approved for use in patients with lung disease). Although the study only produced limited clinical effects this

is likely to be due to the low dose used, and further trials with higher doses are planned for the future (176). IFG has been shown to increase survival and reduce substrate storage in mouse models of neuronopathic GD (177, 178) suggesting that it may be of use in the treatment of patients.

1.1.6.5 Treatment monitoring

Whether or not patients are receiving treatment, they require monitoring by clinicians on a regular basis. This monitoring includes the use of CT scans to assess organomegaly and MRI scans or X-rays to check for skeletal deterioration. Patients also undergo regular blood tests to monitor haemoglobin and platelet levels as well as the plasma concentration of chitotriosidase (ChT), a known biomarker of GD. ChT was first identified as a GD biomarker in 1994 when Hollak *et al.* showed it to be highly elevated in patient plasma samples compared to unaffected individuals and that levels decreased in response to treatment with ERT (179). The exact function of ChT remains unclear but it is known to be highly expressed in the lymph node, lung and bone marrow (180) with the majority of protein expressed by macrophages and monocytic cells (181).

1.1.7 Animal models

In order to study disease it is useful to have animal models which mimic the phenotype seen in humans. To date research into GD has been hampered by a lack of viable models.

Two independent groups attempted to produce a mouse model of GD by targeted disruption of exons 9-11 of the *GBA* gene (182, 183). Although both models resulted in mice with high levels of Gaucher cell infiltration in the liver, spleen and, in one case, the brain; the affected animals all died within 24 hours of birth meaning both models are of very limited use. The affected animals in both studies died of dehydration due to the incorrect formation of skin architecture, not unlike the congenital ichthyosis seen in perinatal-lethal GD.

A subsequent model produced by a Swedish group yielded mice which had a cassette inserted into intron 8 of the *GBA* gene (184). This cassette was flanked by loxP sites enabling excision by Cre recombinase. Mice carrying this insertion were crossed with mice carrying Cre recombinase under the control of a keratinocyte specific promoter (K14). This scheme allows for expression of functional GC which is confined to the skin and is sufficient for rescue of the fatal skin phenotype seen in a ubiquitous knock-out animal. The mice produced in this study developed normally for the first ten days of life and then experienced rapid neurological deterioration leading to continuous seizures and paralysis by two weeks of age. Post-mortem analysis showed widespread neuronal loss and microglial activation (184). This mouse represents a close model of GD type 2 with little visceral pathology; however the absence of GC expression in all tissues means it has utility in investigating the correction of the metabolic defect in type I GD and therefore it has been of use to this project.

Other successful models have used a similar strategy for example Sinclair *et al.* (2007) produced a conditional knock-out in which *GBA* disruption was confined to cells of endothelial and haematopoietic lineages. This model exhibits splenomegaly with Gaucher cells present in the liver and spleen and lives beyond one year of age (185). Models have also been generated carrying specific mutations which correlate with human disease. These include: a mouse homozygous for the L444P mutation which has very limited pathology (186), mice homozygous for N370S which died at birth (187) and mice homozygous for the mutations V349L, D409H and D409V all of which showed extensive GlcCer accumulation in visceral tissues but no accumulation in the CNS (187).

A further model which has been produced recently recapitulates the type I GD phenotype (89). It was created by the targeted knock-out of *GBA* in haematopoietic and mesenchymal stem cells at day two after birth. Mice show significant

hepatosplenomegaly with Gaucher cells present in the liver, spleen, bone marrow and thymus. They also display a skeletal phenotype including osteonecrosis and osteopenia which appears to be due to a defect in osteoblastogenesis as no defect was found in the formation of osteoclasts (89). This represents a very promising model of GD for future experimentation.

1.2 Gene therapy

Gene therapy is an emerging branch of medicine which aims to treat disease by introducing genetic material which will correct the underlying defect. Gene therapy has benefited greatly from significant advances in knowledge of genetics and recombinant technology over the last 100 years; this chapter will describe those advances and the way in which they have led to the advent of gene therapy.

1.2.1 History

In the first half of the 20th century it was observed that injecting a dead, virulent, (S) strain of *Pneumococcus* bacteria into mice in conjunction with an avirulent, living strain (R) led to a fatal bacteraemia caused by the transformation of the R type by some unknown method (188). In 1944, Avery and colleagues determined that the mechanism of transformation was the exchange of DNA between the two bacterial strains (189). This discovery, that DNA from one cell can be used to alter phenotypic characteristics of another, allowed researchers to hypothesise a new method of treating genetic disease – the correction of a genetic defect by supplying a healthy copy of the aberrant gene. This hypothesis gained weight from demonstrations that mammalian cells could also incorporate and retain DNA from exogenous sources (190, 191).

However, the majority of early experiments relied on exposing recipient cells to naked, genomic DNA from the donor source and this only gave inefficient, transient transformation (191-193).

The discovery that malignancies induced by infection with polyoma virus or simian virus 40 (SV40) occur because of the incorporation of viral DNA into the host cell genome (194, 195) suggested the use of viruses as an efficient method of transforming cells. It was at

this time that the first references to 'gene therapy' can be found within academic literature (196) (Figure 1.3).

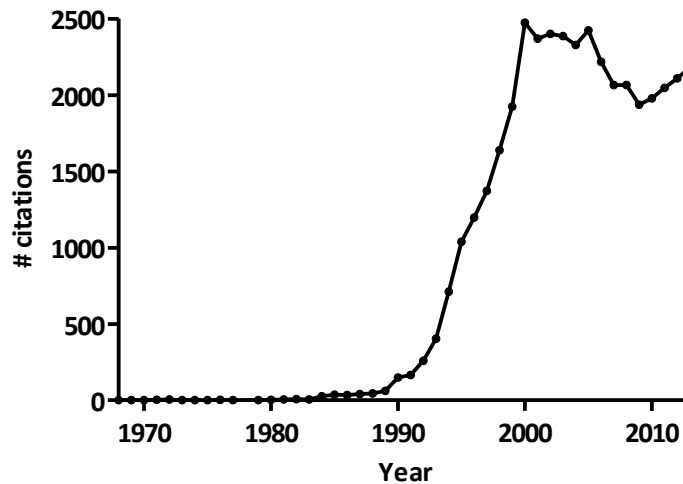


Figure 1.3 Graph showing the number of results from a PubMed search for the term "gene therapy" plotted against year

However, these developments in gene delivery arose before the advent of recombinant genetic technology so researchers were unable to engineer viruses that would deliver specific genes into cells. This changed in 1978 when Tom Maniatis and colleagues announced that they had successfully cloned the gene for β -globin from three eukaryotic species (197). The isolated rabbit gene was subsequently cloned into the viral genome of SV40 and used to infect cells where it was shown to be transcribed into a hybrid virus-mammal mRNA which could be used to produce full-length rabbit β -globin protein (198).

Improved recombinant DNA techniques coupled with increased understanding of viral life cycles allowed several groups to produce replication-deficient retroviral constructs.

When transfected into a cell which was subsequently infected with a replication-competent 'helper' virus, these constructs yielded replication-incompetent vectors as the

engineered genome was incorporated into the viral particle in place of the wild type genome (199-201).

To refine the technology it was necessary to eliminate the requirement for a helper virus. This was achieved by splitting the genes necessary for virus production away from the rest of the viral genome. Concurrent transfection of these genes together with a construct containing the gene of interest and a minimal amount of viral DNA (including a packaging signal) allowed for the formation of infectious viral particles which were capable of transmitting the gene of interest but were replication deficient (202-204). This development will be covered in more detail later in this chapter.

1.2.2 Clinical trials

Some of the first demonstrations of gene therapy *in vitro* used cells derived from patients with Lesch-Nyhan syndrome (hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency). Two papers in the early 1980s from a group in California showed that it was possible to correct *HPRT*^{-/-} fibroblasts (205) and lymphoblasts (206) using a retroviral vector. In the lymphoblast, which is the more disease relevant cell, physiological correction was shown as purine accumulation fell and ATP/GTP ratio normalised (206). The following years saw the publication of papers reporting the genetic correction of patient material from a number of diseases including: adenosine-deaminase linked severe combined immunodeficiency (ADA-SCID) (207), alpha-1 anti-trypsin deficiency (208), Gaucher disease (209) and leukocyte adhesion deficiency (210). As well as different diseases, researchers targeted a variety of cell types including connective tissue (211, 212), renal cells (213), and haematopoietic cells (214, 215).

However, developments faced a setback in the early 1980s when researchers at UCLA conducted an unsanctioned experiment in humans (216). Following their published papers in which they had successfully inserted the genes for thymidine kinase (217) and

dihydrofolate reductase (214) into the bone marrow of mice, the Cline group conducted a trial on two β -thalassemia patients. Bone marrow cells from patients were transduced with recombinant plasmids containing both the β -globin gene and thymidine kinase (a selectable marker) and transfused the cells back into the patients. Neither patient showed any clinical benefit or significant side effects despite being subjected to high level irradiation prior to reinfusion (216). However the work was heavily criticised on administrative and ethical grounds as the construct used had not been tested before the study and it emerged that the research had not been approved by UCLA, where Cline was based, and also that the protocol had been modified from the version approved by the participating institutions in Italy and Israel. As a result, Cline lost his UCLA chair and funding from the NIH (218, 219). It is worth noting however that since no harm came to either of the patients treated in this study, it can be viewed as the first time recombinant DNA was shown to be safe in people which lent strength to the concept of gene therapy as a treatment option.

Despite this setback, gene therapy development continued and in 1992 the first official clinical trial began in the USA. Dr W. French Anderson used gene corrected autologous T-cells to treat a four-year old patient with ADA-SCID (220).

T-cells were harvested by apheresis, transduced with a retrovirus carrying the ADA gene and reinfused to the patient. This was repeated five times over the course of six months. A second patient with a milder disease phenotype was also enrolled in the same trial and at four years post-treatment both patients showed healthy levels of lymphoblastic ADA expression and good clinical outcome even when enzyme replacement therapy was reduced (220). A second trial began in the European Union in 1992 and treated two patients with genetically modified haematopoietic stem cells (HSCs) and peripheral blood lymphocytes. Both patients showed short-term immune reconstitution with expression of

ADA (221). However in both of these studies patients were kept on enzyme replacement therapy and therefore they do not constitute definitive proof of the success of gene therapy. However they did provide more data on the safety of the approach.

Since these first studies, a number of other trials for ADA-SCID therapy have been run which included amendments to try and increase the success rate. These modifications include the refinement of the cytokine cocktail used to culture the stem cells during transduction (222), the use of fibronectin to increase transduction efficiency (223) and the addition of a conditioning regimen to improve engraftment ((224) reviewed in (225)). The most recent set of clinical trials using these adjustments have been run at centres in Italy, the UK and the US and have treated more than 40 patients. Of these, 29 (72.5%) are no longer on ERT and there have been no reported adverse events, making ADA-SCID one of the first diseases to be successfully treated with gene therapy (226-229).

The same technology was adapted to develop a treatment for X-linked severe combined immunodeficiency (X1-SCID), caused by mutations in the IL2RG gene which encodes the γ -chain of interleukin receptors including that of IL2 (230). Trials were run at the Hôpital Necker, Paris and Great Ormond Street Hospital, London treating twenty patients with autologous HSCs transduced by a γ -retrovirus carrying the IL2RG gene. Results showed efficacy in the majority of patients including full immune reconstitution, normal thymic development and clearance of pre-existing infections (231-233). Unfortunately a number of these patients developed T cell leukaemia several months after gene therapy. One patient died as a result but the rest responded to chemotherapy and went into remission (234). Genetic analysis determined that the reason for the uncontrolled expansion of T cells was an insertion of the viral genome close to genomic loci containing proto-oncogenes, usually the LMO2 locus but also CCND2 and BMI1. The strong viral enhancer element in the viral backbone upregulated expression of genes at these loci which

resulted in increased proliferation and survival of cells containing these insertions (234-236).

Clonal expansions were also observed in gene therapy trials for chronic granulomatous disease (leading to the death of one participant) (237, 238) and β -thalassemia (239) using γ -retroviruses although in these cases the expansions were found to have therapeutic benefit.

In 1999, the gene therapy field suffered a major stumbling block when researchers at Pennsylvania State University working on ornithine transcarbamylase) deficiency treated an eighteen-year old boy named Jesse Gelsinger as part of their phase I clinical trial (240). The previous seven patients had been treated with escalating doses of intravenously administered virus and had experienced minor side effects (transient thrombocytopenia, myalgia, fever) but no serious reactions that might suggest a danger to future participants (241). However 18 hours after treatment Jesse Gelsinger began to exhibit signs of liver failure and 35 hours post-administration, he fell into a coma from which he never recovered, dying 98 hours after treatment despite the best efforts of the research and other medical teams (240). Subsequent investigations revealed a number of issues with the study which has led to revised policy on the reporting of adverse events in pre-clinical studies as well as the involvement of trial staff with financing organisations (242, 243).

Despite the many challenges faced during the development of gene therapy, researchers have persevered. Figure 1.4 shows the number of clinical trial records in the PubMed database (<http://www.ncbi.nlm.nih.gov/pubmed>) returned by a search for the term “gene therapy” over time. This graph shows clearly the effect that the death of Jesse Gelsinger had on the gene therapy community, with the number of reports filed falling from forty in 1999 to twenty-four in 2002. This drop reflects the public loss of trust in

clinical research after the widespread reporting of his death, as well as restrictions placed on a number of organisations while investigations were being conducted. The graph also shows a second fall in the number of clinical trials being conducted which corresponds with the reporting of T-ALL development in participants of the X1-SCID trial in 2003. Nevertheless, gene therapy continued to persevere with 25 records deposited in the PubMed database in 2013 and 120 trials in progress according to the Journal of Gene Medicine. The Journal of Gene Medicine collates the number of gene therapy trials and this data shows the same trend as the PubMed search with a steady increase in the number of records despite a drop in the number of records around 1999 and 2003.

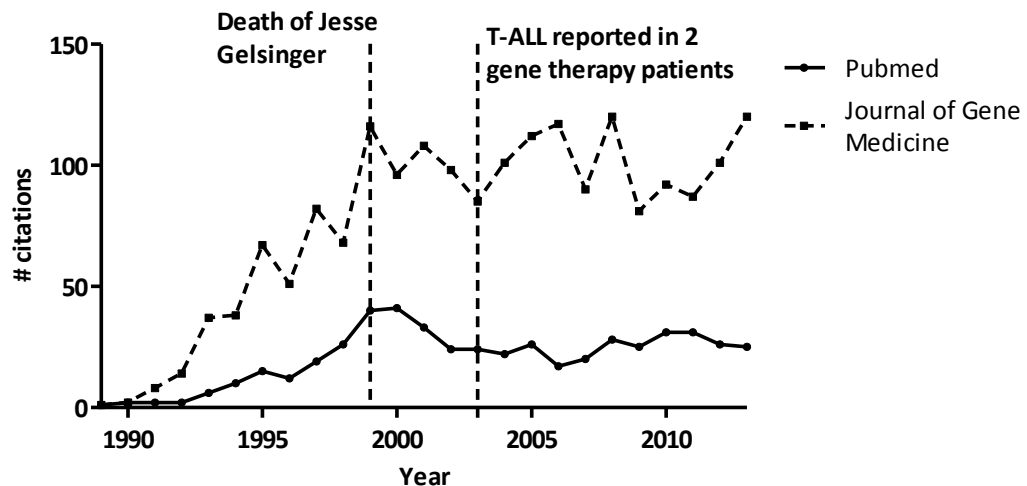


Figure 1.4 The number of gene therapy trials reported by two sources from 1989-2013

Pubmed search for the term "gene therapy" restricted to records labelled as "clinical trials" (solid line) and the number of trials approved worldwide according to the Journal of Gene Medicine (dashed line).

It is worth noting at this point that although the development of gene therapy has faced a number of setbacks, the development of a new treatment is rarely smooth. For example,

an early review of bone marrow transplantation as a treatment strategy showed that 152 of 203 patients had died after the therapy leading to widespread discontinuation of the practice (244) before going on to become the standard of care for a number of conditions.

1.2.3 Vectors

A number of different virus types have been developed as vectors for gene therapy including: retroviruses (and lentiviruses), adenoviruses (Adv), adeno-associated viruses (AAV) and herpes viruses. Table 1.3 summarises the vector types available.

A variety of virus genera have been developed as vectors because of the differing requirements for treating the large variety of diseases targeted by gene therapy and the following pages will explain some of the differences between the vector types and their advantages and disadvantages in a clinical setting.

One of the first considerations when choosing a vector type is the amount of genetic material which can be incorporated into the virion. AAV is the smallest gene therapy vector and has a capacity of 5kb which must include the transgene in addition to any other genetic components such as promoter or enhancer elements as well as any viral structures and therefore limits the utility of the vector as it cannot be used to transport large genes (245).

An example of how the size of insert can affect vector choice comes from research into gene therapy for muscular dystrophy (MD). The gene responsible for causing both Becker and Duchenne muscular dystrophy is the *dystrophin* gene which has a cDNA of 11kb and this has hindered gene therapy for MD (246).

Another feature which must be considered when choosing a vector system is the outcome of cellular transduction. Some vector systems such as retroviruses and AAV are able to integrate their genetic material into the host chromosome enabling maintenance and replication of the transgene within the cellular genome. This is important for the treatment of many conditions as it allows for the permanent correction of a genetic defect. It is of particular use when treating stem cells as it ensures that the therapeutic effect will not be lost as cells divide and differentiate. However there is a risk that integration can lead to insertional mutagenesis which can have an oncogenic effect as has been seen in a number of clinical trials (234-238).

Some vector types including adenovirus, herpes virus and non-viral vectors are unable to mediate this integrative effect and the transgene remains outside the genome as an episome. Although episomal DNA will be lost if a cell divides, non-integrating vectors are of use when treating post-mitotic cells such as skin or neurones as the lack of integration removes the risk of insertional mutagenesis and the transgene will persist for the life of the cell which is often of sufficient length to provide relief from the disease.

A number of viral vectors that have been developed to date have been derived from known human pathogens. In addition to the possibility that these vectors could be pathogenic themselves, this also introduces the risk that patients will have pre-existing immunity to the vector. This is a particular problem with Adv and herpes virus vectors as well as some AAV serotypes (247). Pre-existing immunity to a vector can result in its eradication from the body by the immune system before a clinical effect can be produced but it can also result in severe complications for the patient as seen in the case of Jesse Gelsinger (240). Vectors which have an immunogenic effect are also of limited use to researchers as they can only be used once for each patient and so the option of a repeat treatment is not available (although this can be overcome to a certain extent by changing

the serotype of virus when using AAV or Adv). Additionally, the issue of immunity is only a problem when vectors are administered directly to the patient; in therapies where patient cells are treated *ex vivo*, as is often the case when treating haematopoietic stem cells, the virus is removed from the cells prior to their reinfusion to the patient reducing the likelihood of the virus and the host immune system coming into contact with one another.

With these considerations in mind, a range of vector types have been developed. This project uses a subset of retroviral vectors known as the lentiviruses which will be covered in detail but a brief overview of the other vector types will be given here.

Adenoviruses are a family of over 50 serotypes which commonly cause respiratory and intestinal tract infections. They are double-stranded DNA viruses which have an insert capacity of up to 36kb (serotype dependent). Adenovirus predominantly targets epithelium but the native receptor is expressed in almost every tissue. Expression of the receptor is greatly upregulated on malignant cells making Adv ideal for cancer gene therapy (248). However this significant benefit for Adv vectors is countered to a certain extent by the significant immunogenicity of the virus type. This is due to both the vector capsid and the residual viral genes which are expressed, albeit at low levels, in transduced cells (249). Not only does this high immunogenicity reduce the potential for repeating a treatment, it also limits the duration of transgene expression which can be useful where only short-term expression is required or the target cells are post-mitotic but is of little use where the goal is permanent correction. Pre-existing immunity also limits the initial dose that can be administered as lower doses reduce the risk of stimulating an immune response.

The smallest viral vector to be developed is derived from the adeno-associated viruses (AAV); non-pathogenic, single-stranded DNA viruses with a maximum capacity of 5kb (245). This small size in addition to relatively high immunogenicity and high levels of pre-existing immunity can limit utility in gene therapy to a certain extent (250) but it remains a popular virus type for a number of reasons. There are at least eleven different serotypes of AAV which all have distinct antigenic profiles. This array of serotypes of AAV gives a wide range of tropisms allowing vectors to be designed on the basis of the intended target. For example AAV8 has a strong tropism for liver transduction (251) whereas both AAV8 and AAV1 show a preference for muscle targeting (252). Additionally, it is possible for researchers to 'pseudotype' AAV vectors, using the genome of one serotype in combination with the capsid of another to alter the tropism of the vector (253). This strategy can also be used to overcome the problem of repeat administration of a vector which has significant immunogenicity as changing the capsid protein would provide resistance to neutralising antibodies produced after the first administration.

One of the most significant discoveries in AAV gene therapy was the revelation that AAV9, or virus pseudotyped with the AAV9 capsid, is able to cross the blood-brain barrier and can mediate wide-spread nervous system transduction (254, 255). This is of great benefit to gene therapy researchers as it is currently the only vector system able to transduce neuronal tissue without the need for intracranial delivery.

Herpes simplex virus (HSV) type 1 has a 152kb, single-stranded DNA genome resulting in vectors which can incorporate very large genes. It is also a highly infectious virus type with a broad host range (despite the wild type virus being predominantly neurotropic) and is able to infect dividing and non-dividing cell types. All of which makes HSV-based vectors a very promising area of gene therapy research.

Not all gene delivery methods rely on viral vectors, particularly when integration is not required and the target tissue is easily accessible. Non-viral vectors often consist of a positively charged polymer (for example: polylysine or polyethylenimine) or lipid (such as lipofectamine) which condenses around the DNA and is able to fuse with the plasma membrane enabling delivery of the genetic material. Although naked DNA can be delivered to cells using methods such as electroporation or the gene gun, encapsulation within a polymer increases the stability of the material allowing for more widespread distribution. Non-viral vectors have some significant advantages over viral agents as they have unlimited capacity so are able to transport very large genetic elements. They also have a very low immunogenic profile so that the risk of pre-existing immunity or inducing an immune response is greatly reduced compared to a viral vector and re-administration of a treatment is usually permissible. However the efficiency of non-viral methods is quite low compared to viruses, especially *in vivo* which has limited their use.

	γ-retrovirus	Lentivirus	Adenovirus	Adeno-associated virus	Herpesvirus	Non-viral
Packaging capacity	9kb	10kb	30kb	5kb	100kb	Unlimited
Transduction of non-dividing cells	No	Yes	Yes	Yes	Yes	Yes
Integration into host genome	Yes	Yes	No	Yes	No	No
Duration of expression	Long	Long	Short	Long	Short	Short
Pre-existing immunity	No	No	Yes	Some serotypes	Yes	No

Table 1.3 Viral types used as gene therapy vectors adapted from (256)

1.2.4 Lentiviruses

Many of the earliest gene therapy experiments used vectors developed from the Retroviridae; a family which is characterised by a single-stranded RNA genome which is reverse transcribed into DNA within the host cell and subsequently integrated into the host genome (257-259). The Retroviridae is a large family consisting of seven genera including the alpharetroviruses, gammaretroviruses, lentiviruses and spumaviruses most of which have been developed as gene therapy vectors. The earliest clinical trials focused on the use of the gammaretroviruses but in recent years the focus has shifted towards lentiviruses such as the vectors used in this report.

One of the principal reasons the Retroviridae were chosen as vectors is the integration of the viral genome after transduction of target cells. Once DNA is integrated into the host genome, it will replicate with the cell and so the delivered gene will be maintained throughout cell division and differentiation (259, 260). In this way correction of cells can be seen as permanent. This is of significant benefit to gene therapy researchers treating progenitor cells as it means that a treatment may only need to be performed once.

However, factors including the association of retroviral transduction with the development of leukaemia seen in some trials (234-236) led researchers to look for alternative viral vectors. One such alternative was found in the lentivirus family, a subset of retroviruses which include the human immunodeficiency virus 1 (HIV-1) which preferentially integrate within actively transcribed genes (261) unlike γ -retroviruses, which have a preference for integrating close to transcription start sites (261-263). There is also evidence that γ -retroviruses but not lentiviruses have integration hotspots within the region of proto-oncogenes which increases their potential for insertional mutagenesis (264).

1.2.4.1 HIV

This section will give some general background to wild type HIV biology and explain the changes required to develop gene therapy vectors.

Genome

The wild type HIV-1 genome can be split into two types of sequence: coding and non-coding. Coding sequences include the three major HIV-1 genes – *env*, *gag* and *pol* – in addition to genes encoding accessory and regulatory proteins. The non-coding sequences consist primarily of the 3' and 5' untranslated regions (U3 and U5 respectively) together with primer binding sites for reverse transcription, and a packaging signal (265).

The genome is transcribed by the host cell RNA polymerase II (PolII) which is recruited to the TATA box within the 5' long terminal repeat (LTR; Figure 1.6) (265) by the viral TAT protein and the host cell protein P-TEFb (266, 267) and results in multiple mRNAs. Translation of the unspliced viral mRNAs occurs at host ribosomes and results in a number of polyproteins which require further processing to yield the functional viral proteins (268-270).

Env

The HIV envelope protein (Env) consists of two parts – surface and transmembrane units (271, 272) which associate to form the structure which is responsible for mediating cellular entry (273, 274). The Env complex is only weakly associated with the virion which has allowed for pseudotyping of lentiviral vectors with alternative proteins. Pseudotyping has allowed the creation of lentiviral vectors carrying the envelope proteins of the vesicular stomatitis virus, murine leukaemia virus and herpes simplex virus among many others (275).

Gag-Pol

The major structural proteins of a lentiviral virion are encoded by the *gag* and *pol* genes which overlap by 205 or 241 nucleotides (268, 276). Transcription of the *gag* and *pol* genes yields two polyproteins, Gag (55kDa) and Gag-Pol (160kDa), due to ribosomal slippage which allows the host ribosomes to read through the stop codon between the two genes (Figure 1.5) (268, 269). It appears

that ribosomal slippage occurs infrequently as the ratio of Gag to Gag-Pol production in an infected cell is approximately 20:1, analogous to the ratio of the same proteins in other retroviruses. This 20:1 ratio appears to be important for maintaining the infectivity of viruses (277).

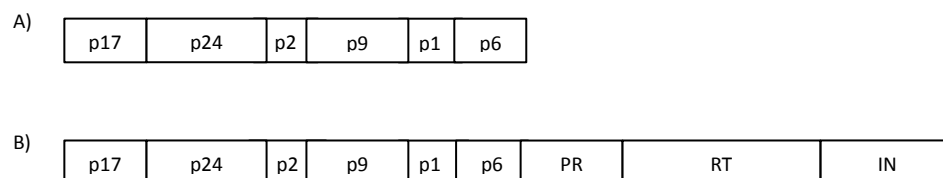


Figure 1.5 Structure of the Gag and Gag-Pol polyproteins of HIV-1

Schematic showing the two polyproteins translated from the gag and pol genes of HIV-1. A) Gag polyprotein B) Gag-Pol polyprotein p17: matrix (MA), p24: capsid (CA), p2/p1/p6: spacer proteins, p9; nucleocapsid (NC), PR: protease, RT: reverse transcriptase, IN: integrase

The cleavage of the polyproteins is an essential step in the production of a mature virion as without it, the viral core cannot form (278) and the RNA is not adequately stabilised (279) resulting in non-infectious particles (280). Cleavage is performed by the HIV-1 protease which is released from the Gag-Pol polyprotein by autoproteolysis and goes on to cleave the rest of the protein (281).

The components of the Gag polyprotein (Figure 1.5A) are structural proteins which are important during viral assembly including the three main proteins – capsid (CA), nucleocapsid (NC) and matrix (MA). All three are essential proteins due to their role in forming the virion; NC and CA are involved in encapsulating the genome (282-287) while MA targets the complex to the plasma membrane where the full molecule is formed (288, 289).

In addition to CA, NC and MA, the full length Gag-Pol polyprotein (Figure 1.5B) yields two other enzymes which are essential for viral function: reverse transcriptase and integrase.

Reverse transcriptase

The first of these, reverse transcriptase (RT), is responsible for the conversion of the RNA genome to double stranded DNA which can be processed by the host cell. The occurrence of the reverse transcriptase event was hypothesised by Howard Temin in 1964 (290) and was found to be due to the action of a viral protein in 1970 by two separate groups (257, 258). The protein itself functions as a heterodimer composing of a 66kDa subunit and a smaller, 15kDa subunit (291) which are both products of Gag-Pol cleavage. The complete enzyme has both polymerase and RNase activities both of which are essential for reverse transcription to occur (292). The process of reverse transcription itself has taken several years to elucidate but is now relatively well understood.

The HIV-1 genome is plus-stranded meaning that both strands of DNA are synthesised from the same molecule of RNA using a host cell tRNA (Lys3) as a primer (293, 294). The polymerase activity of RT uses nucleotides present in the host cell, and the RNA template to extend the primer and begin synthesis of the DNA strand. After polymerase extension, the RNase H activity of RT degrades the RNA portion of the RNA-DNA complex produced (295). Production of the second DNA strand is mediated by a short section of the RNA genome known as the central polypurine tract (cPPT) which is resistant to RNase degradation and so is able to act as a primer for second strand synthesis (296).

The final product, called a provirus, is larger than the original RNA genome as each end contains both U3 and U5 regions in a combination known as a long terminal repeat (LTR) (297).

Each viral particle produces one provirus (297) and reverse transcription products peak around twelve hours post-transduction with integrated DNA being detected by twenty four hours after infection (298).

Integrase

RT is responsible for the production of the DNA provirus but it is the integrase (IN) protein, a 32kDa protein found at the N-terminus of the Gag-Pol polyprotein, which catalyses the integration of the

provirus into the host cell genome (259). Within the cell IN localises to the nucleus as it contains a nuclear localisation signal (NLS) (299) and it binds to DNA through the C-terminus (300).

To begin the process of integration a tetramer of IN proteins binds to both LTRs of the newly synthesised viral DNA (301) and associating with other cellular factors including LEDGF/p75 and Ku70 to form the pre-integration complex (PIC). The PIC mediates nuclear entry by a mechanism which remains unclear but which is likely to involve proteins at the nuclear envelope such as importin- α (302), nucleoporin 153 (303) and ADAM10 (304).

Once nuclear entry has been achieved, IN cuts the host chromosome leaving a 5bp overhang and simultaneously joins the 3' end of the viral DNA to the 5' phosphate group of the host genome (305). Host cell enzymes then repair the cut DNA and fill in the overhangs caused by IN (306, 307)

Accessory proteins

In addition to the major proteins transcribed from the *env*, *gag* and *pol* genes, HIV-1 requires two other proteins – regulator of expression of virion proteins (Rev) and transactivator of transcription (TAT) both of which are involved in the regulation of HIV gene expression.

Rev acts post-transcriptionally by controlling nuclear export of the viral mRNA allowing for translation of viral proteins (308). It is able to mediate this effect as the Rev protein binds to a motif within the RNA known as the Rev response element (RRE) and is exported from the nucleus by virtue of a nuclear export signal within the protein itself (309, 310).

TAT functions to increase transcription of the viral genome. It binds to a site in the RNA known as the transactivation-responsive region (TAR) (311) which enhances elongation of the transcribed mRNA (312). The TAT protein is secreted from, and taken up by, infected cells in an autocrine fashion (313, 314) through a protein transduction domain which can be used to engineer the transduction of other proteins into a cell (315-317).

In addition to the essential proteins described above, the HIV-1 genome encodes a number of other accessory proteins which have a variety of functions but no intrinsic enzymatic activity. These include nef, vif, vpr and vpu all of which can be deleted from the genome without any significant effect on the ability of the virus to act as a vector (318, 319).

Viral structure and assembly

A mature viral particle contains two copies of the RNA genome as well as the host cell tRNA Lys3 required for reverse transcription and the viral IN, RT, PR and Gag proteins transcribed in the producer cell. The virion is encapsulated by a coat of the cell's plasma membrane which contains molecules of the assembled Env protein. Some of the accessory proteins and some cellular factors are also packaged into the virion but in much lower quantities (320).

The first step in viral assembly is dimerization of the RNA genome which is driven by the NC protein (282, 284), which recognises the viral packaging signal (Ψ) (321). The full length Gag polyprotein also associates with the RNA genome through the integral NC sequence as well as the uncleaved CA C-terminal domain (322). The MA portion of Gag is then able to bind to PIP2 in the plasma membrane and this facilitates the localisation of the Gag-RNA complex to the cell membrane (323).

The mechanism of Env incorporation into the virion remains unclear. It is known that the cytoplasmic domain of gp41 interacts with MA (324, 325) but the nature and necessity of this interaction remains unclear (326).

Once the viral components are assembled at the membrane, the virion is produced by 'budding' off the cell, a process which may involve the host cell 'endosomal sorting complexes required for transport' pathway (327, 328).

Once budding has occurred, or possibly concurrently, the Gag and Gag-Pol polyproteins which have been incorporated undergo cleavage by the viral PR and the virion takes on the characteristic structure of a retrovirus. NC condenses around the genome, CA forms the 'Fullerene cone' structure

which encapsulates the RNA, RT and IN molecules (329, 330) whereas MA appears to remain associated with the lipid envelope (331, 332). This process is called maturation and it is the final step in viral production.

1.2.4.2 Lentiviral Vectors

Several important steps had to be taken in order to develop lentiviral vectors suitable for use in gene therapy from wild type HIV.

One of these steps was the development of so-called 'self-inactivating' viruses by the deletion of promoter/enhancer sequences within the LTR so that vectors can be regulated by external elements. The endogenous retroviral promoter and enhancer elements are contained within the U3 region of the virus and function only after reverse transcription when they have been translocated to the 5' LTR (333). Yu *et al.* (1986) introduced a 299bp deletion within U3 which removed both the enhancer and the promoter elements and replaced them with an internal promoter to drive transgene expression (334). This has proved a hugely important development as deletion of the endogenous promoter has allowed researchers to control transgene expression by choosing a relevant internal promoter. For example, a gene or tissue specific promoter could be used to restrict expression to a particular cell lineage (225) or tissue (335, 336). Additionally, recombinant promoter elements such as ubiquitously acting chromatin opening element (337, 338) or the β -globin locus (225) can be used to give sustainable, high levels of gene expression. Many early experiments used viral promoters such as the spleen-focus forming virus (SFFV) promoter (237) or the endogenous retroviral LTR promoter (220) as these are expressed in a wide number of cell types and usually at high levels. Using a strong promoter allows for high levels of protein expression even when transduction and rate of genome integration is low. However viral promoters fell out of favour as they are prone to methylation by the host cell which leads to gene silencing (339, 340) and because many contain enhancer elements which increase the risk of insertional mutagenesis (235, 237).

A further development came about because many viral proteins are present in the viral particle itself and do not require transcribing in the host cell. Therefore the genes encoding these proteins can be removed from the viral genome and supplied *in trans* in a packaging cell. Supplying genes *in trans* has two advantages: first it reduces the size of the viral cassette, allowing for the packaging of larger therapeutic nucleic acids; secondly, minimising the viral genome reduces the possibility that the vector will recombine with any viral sequences present within the host genome and produce a pathogenic or replication competent virus (341).

Figure 1.6 shows the development of lentiviral vectors from the wild type HIV-1 genome by sequentially splitting the genome into separate plasmids. The first generation vectors are described in Naldini *et al.* (1996) and carry the majority of the viral genome on three plasmids (342).

The main packaging construct contains the *gag* and *pol* genes which encode all the necessary enzymes and regulatory sequences including Vif and Vpr. It also contains a defective version of the *env* and *vpu* genes and a 5' UTR from which the packaging signal has been deleted but the splice donor preserved. The 3' LTR has been replaced by a polyA signal which eliminated the sequences required for packaging, reverse transcription and integration of the plasmid (342).

The second plasmid contains the gene encoding the envelope protein under the control of the CMV promoter. Changing the envelope gene included in the plasmid provides an opportunity to pseudotype the vectors (342, 343); altering the protein coat in order to direct the tropism of the virus. In the original paper, as in many subsequent studies (including this project), the envelope gene used encodes the vesicular stomatitis virus glycoprotein (VSV-G) which increases the stability of virus particles as well as providing a wide tropism (344).

The transfer plasmid (pHR') contains the gene to be transferred to the target cells, as well as 350bp of the *gag* gene which, in combination with a part of the *env* gene spanning the RRE sequence, increases packaging efficiency and limits transcription to occurring only in the presence of TAT (as the

TAT binding sequence is in the LTR) and Rev proteins generated by the packaging construct. The *gag* sequence also contains the packaging signal allowing for incorporation into viral particles. This third plasmid also has a full length 3' LTR allowing for reverse transcription and integration (342).

In order to generate new vectors containing even less of the original viral genome, researchers performed deletion studies on the packaging plasmid which showed that the accessory genes, *vif*, *vpr*, *vpu* and *nef* as well as the entire *env* gene can be deleted from the plasmid without adverse effects on titre or viral infectivity (318, 319). Vectors produced from this reduced packaging cassette are known as second-generation lentiviral vectors (Figure 1.6).

Further efforts to improve the biosafety of lentiviral vectors have yielded a third generation of vectors in which the *rev* gene has been separated onto a fourth plasmid and deletion of the *TAT* gene was made possible through the use of a strong, constitutive promoter within the chimeric 5' LTR used on the third generation transfer plasmid (345).

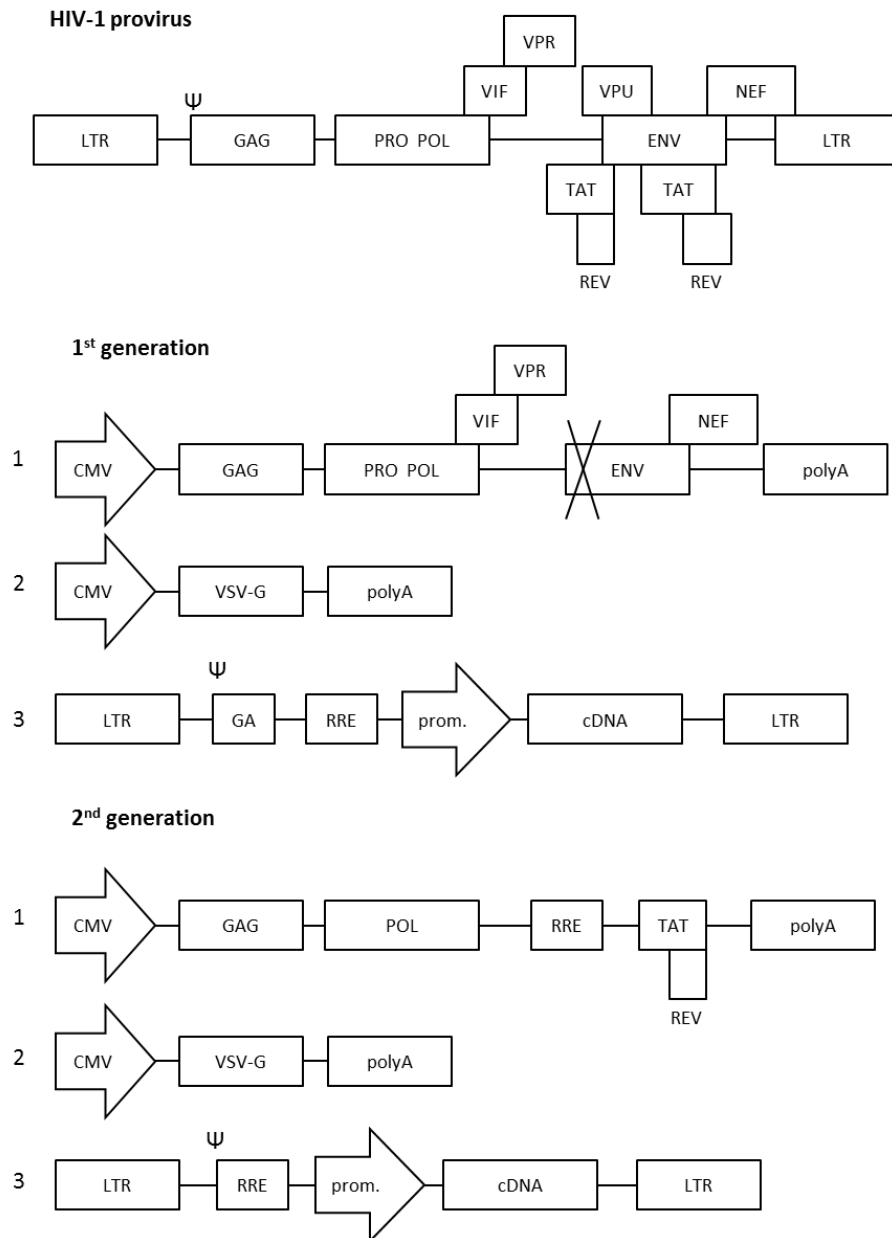


Figure 1.6 Development of first and second generation lentiviral vectors from the HIV-1 provirus

LTR: long terminal repeat; GAG: polygene encoding virion structural parts; PRO POL: polygene encoding viral enzymes; VIF, VPR, VPU, NEF are viral accessory proteins; ENV: viral envelope gene; TAT, REV are regulatory sequences; CMV: cytomegalovirus promoter; polyA: polyadenylation signal, VSV-G: vesicular stomatitis virus glycoprotein, GA: attenuated GAG sequence; prom: promoter ; cDNA: complementary DNA sequence (transgene); Ψ : packaging signal. For 1st and 2nd generation vectors, plasmids 1 and 2 are supplied *in trans* in the packaging cell line and only plasmid 3 is incorporated into the virion.

1.2.5 Protein transduction domains

Many conditions which are a target of gene therapy are restricted to a particular cell type or lineage and therefore it is sufficient to modify only this tissue. While this is likely to be the case for GD as demonstrated by the fact that macrophage targeted ERT is able to relieve the majority of symptoms; it may also be beneficial if there is more widespread delivery of GC. This is because *GBA* is a housekeeping gene and as such is expressed ubiquitously albeit at low levels. There are two methods to achieving systemic or widespread protein distribution, one of which is to administer vector systemically allowing it to target all tissues. However this method risks stimulating an immune response within the recipient as well as requiring higher vector doses and giving reduced control of the actual tissues modified.

An alternative method of achieving widespread correction is to focus on increasing the extent of protein rather than gene, distribution. One way is to fuse the protein, which has an endogenous secretion signal, to a protein transduction domain (PTD) which would allow any secreted protein to penetrate unmodified cells in the environment. PTDs often function through receptor-independent mechanisms making them less specific and allowing them to target a wide variety of cell types which is ideal in this scenario.

One of the best characterised PTDs is that of the HIV-1 transactivator of transcription (TAT) protein domain. The PTD from the TAT protein has been shown to work by stimulating protein uptake by lipid raft-mediated endocytosis when the fusion protein is greater than 30kDa (346, 347). This method of protein transduction is ideal for lysosomal proteins such as GC because once the protein has been endocytosed it becomes trapped in the endosome which progresses through the cell and fuses with vesicles which contain lysosomal proteins and have low pH. This becomes the mature lysosome which is the site of action of GC (4, 13, 14).

A previous study demonstrated that in an ERT setting, addition of the TAT PTD to the C-terminus of GC results in a significant increase in the amount of protein taken up by fibroblasts in culture

compared to both imiglucerase and wild type GC (315). The same paper showed that fusion of the PTD to the N-terminus of the protein abolished protein expression. A subsequent study demonstrated the presence of two furin cleavage sites within the PTD which presents a possible explanation for the lack of expression from the N-terminal fusion (316). The authors were able to show that changing the amino acid sequence could remove the furin cleavage sites while maintaining the cell penetrating properties of the domain. This modified version of the PTD (mTAT) is used in this project in addition to the WT version (WT TAT) to compare the effect of PTD fusion to GC.

The TAT PTD was the first entity to be described as a mediator of protein transduction (314, 348, 349) but since those early studies, many other domains have been described. These include: Antp, from the *Drosophila* Antennapedia protein (350); VP22, found in Herpes Simplex virus (351); and polyarginine (352) or polylysine domains (353, 354). One of the most important factors that make for a successful PTD is a positive charge so that the domain can interact with the anionic cell membrane allowing for the uptake of the associated protein (347). For this reason most PTDs contain a high proportion of the basic amino acids arginine and lysine.

Studies comparing the different forms of PTD have repeatedly shown the polylysine and polyarginine domains to be very effective at enabling protein transduction but it has also been suggested that they may be more toxic than other sequences with Antp being at the opposite end of both spectra (354-356). This study used the TAT PTD because of previous work which has shown it to be capable of mediating the transduction of GC and other lysosomal proteins into various cell types (315, 317, 357, 358) but it would be worth investigating other domains particularly those which have higher efficiency such as Antp.

One potential limitation of PTDs which must be considered is the need for the protein to escape the endosome after internalisation. However this is not relevant for GC as its site of action is the

lysosome which is the ultimate location of endocytosed molecules and it is in fact of great benefit that the protein is delivered to the lysosome without the requirement for extra adaptations.

1.3 Gene therapy for Gaucher disease

As discussed above, gene therapy has been used to treat a number of monogenic disorders and this project aims to develop gene therapy for Gaucher disease.

There are a number of reasons that GD is being considered as a candidate for gene therapy. Firstly, it is a monogenic disorder and the gene responsible has been well characterised so that researchers have a good idea of the tissues which need to be targeted and the level of expression necessary to correct the disease (studies have shown 11-15% enzyme expression to be sufficient for correction *in vitro* (54) and 1-10% engraftment of WT HSCs *in vivo* (359)). Secondly, correction of the disease has been seen following allogeneic haematopoietic stem cell transplantation (HSCT) with patients showing reversion of skeletal manifestations (360) and neurological stabilisation of type III disease if performed sufficiently early in life (100, 101, 361). However, these observations are based on very limited clinical data as HSCT has never been used as a routine treatment option and very few patients have undergone transplantation since the advent of ERT (106).

The fact that HSCT can be a curative treatment for Gaucher disease serves as proof of the principle that gene therapy is a suitable option for treating GD. *Ex vivo* gene therapy would involve treating the patient with a genetically corrected autologous haematopoietic stem cell graft which is potentially safer than HSCT from an allogeneic donor because there is reduced risk of rejection or graft versus host disease (GVHD). GVHD occurs when transplanted T cells mount an immune response against the recipient tissues and is a major complication of HSCT with symptoms including skin rashes, gastrointestinal disturbances and liver failure (362). This risk should be eliminated by using autologous HSCs.

Before the development of ERT, there was significant interest in gene therapy as an option for GD because of the limitations of HSCT and the shortage of suitable donors. This interest began to wane after ERT became a licensed treatment in the early 1990s and was proved to be effective and

popular with patients, with the result that there have been relatively few gene therapy studies published in the last 20 years.

The studies published in the pre-ERT era, used a range of vector types, predominantly gammaretroviruses (209, 363-365) although there are occasional reports using lentiviruses (366, 367) or adeno-associated virus (368, 369). In addition to using different vector types, papers also focussed on a number of different cell types including: fibroblasts (370), lymphoblasts (370), myoblasts (371) and, most relevantly for gene therapy, haematopoietic stem cells (209, 363-366).

Two papers, Correll *et al.*, (1992) and Ohashi *et al.*, (1992), both showed that when murine HSCs were transduced with a γ -retrovirus carrying the *GBA* cDNA and transplanted into recipient mice, supranormal levels of enzyme activity could be found in macrophages of the BM and spleen up to seven or eight months post-transplantation (363, 372). This study showed that transduction of HSCs with *GBA* can give rise to corrected macrophages *in vivo* but used an early vector-promoter system which is less clinically relevant than a lentivirus and also was not a disease model as in both cases, the donor and recipient mouse strains were *GBA*^{+/+}. A similar study which also used WT mice as both donor and recipient showed that lentiviral vectors could also mediate long-term reconstitution up to 6 months post-HSCT and even after a secondary transplant (366).

In 1998, an American group initiated a clinical trial for gene therapy using *ex vivo* transduction of CD34+ cells with a retroviral vector in three patients (373). This study showed very low gene transfer into the target cells and one day after reinfusion, the cDNA sequence could not be detected in any of the patients. Some gene marking was seen in one patient three months post-infusion indicating short-term survival and expansion of the transduced cells, but the level of cDNA detected was low and was lost six months after treatment. None of the patients had any significant increase in GC expression or activity at any point during the study (373). It is worth noting that the study protocol did not include a conditioning step before the administration of transduced cells. Conditioning involves the administration of chemotherapeutic agents to suppress or destroy the recipient

haematopoietic system. The lack of a conditioning step in the previous study protocol could mean that if the corrected cells have no competitive advantage in the bone marrow environment, it is likely that they were outcompeted by the unmanipulated HSCs and were therefore lost. It is probable that any future gene therapy trial for GD would need to contain a conditioning step and that that conditioning would probably need to be complete (Adrian Thrasher: personal communication). This study also highlights the need for higher levels of transduction than were achieved in this paper.

One of our reasons for investigating gene therapy as a potential treatment for GD is the skeletal disease which remains the main cause of morbidity in type I patients on ERT. In many cases bone disease is refractory to ERT (153, 157) whereas HSCT has been shown to be curative for a number of skeletal complications (99, 100). It is likely that the ability of HSCT to ameliorate skeletal disease to a greater degree than ERT is due to direct correction of the osteoclast, a member of the haematopoietic lineage (Figure 1.7) (374). There has been very little research into the use of gene therapy within the context of the osteoclast and what work there is has tended to focus on the transduction of preosteoclasts, rather than true stem cells (375). There is one recent report, however, which showed that by targeting HSCs as opposed to more committed cells, levels of transduction are greatly improved (376) which is a very promising result for the potential of gene therapy to treat skeletal disease. There have also been equally promising results from studies which have been developing treatments for the gene therapy of another osteoclast disorder, osteopetrosis (377).

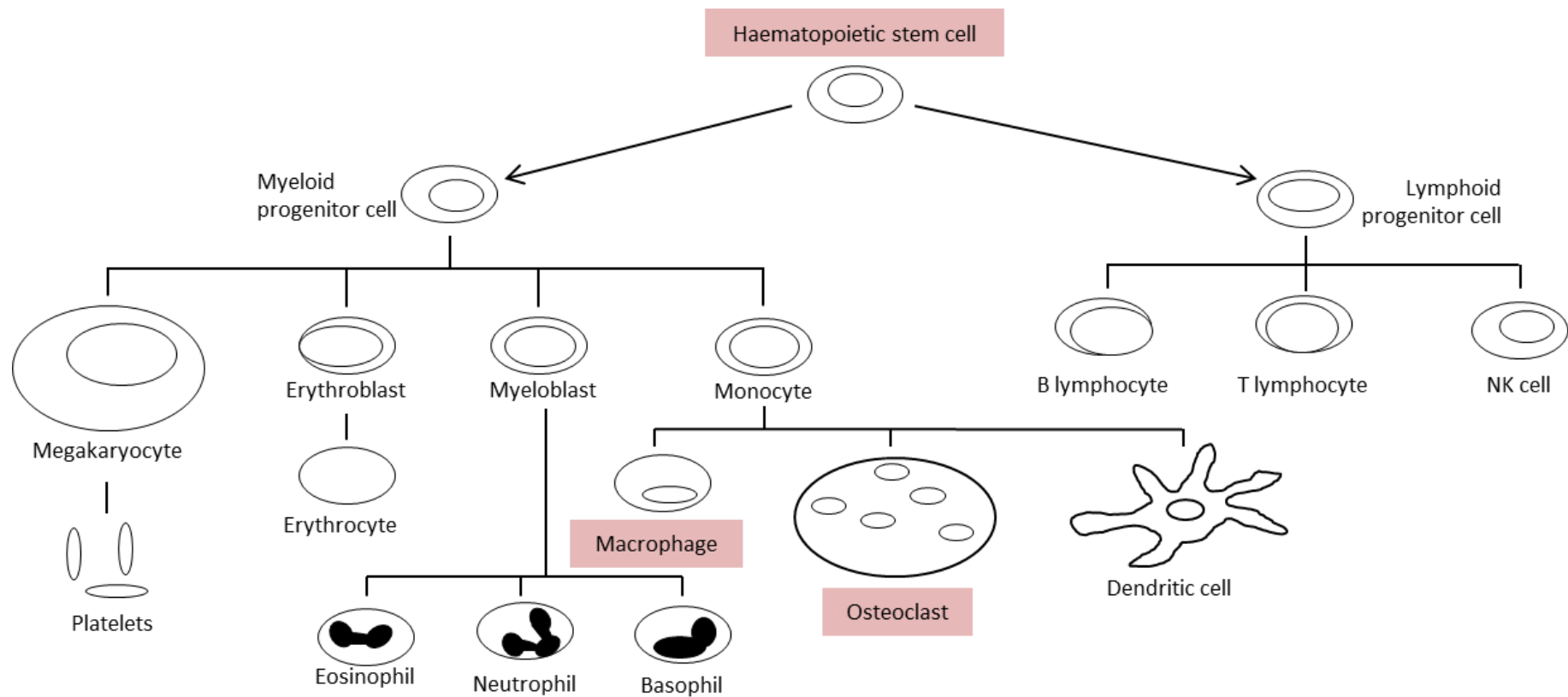


Figure 1.7 Cells of the haematopoietic lineage showing differentiation of the macrophage and osteoclast from the HSC.

Both of the major cell types involved in GD, the macrophage and the osteoclast, differentiate from HSCs meaning that a gene therapy strategy targeting the HSC could be successful in treating both cell types directly.

1.4 Summary and Aims

We hypothesise that lentiviral vectors will provide a suitable method of mediating *ex vivo* gene therapy for GD.

In chapter 3 of this study we aim to develop vectors which are capable of correcting the genetic defect seen and which will be tested using patient-derived cell lines as well as tissue harvested from a *GBA*^{-/-} mouse model (184).

The majority of GD patients are currently treated with an enzyme replacement therapy which directly targets the macrophage (109) but as *GBA* is a housekeeping gene with some expression in almost all tissues of the body (378-380); it may be preferable to develop a therapy which is capable of restoring expression to tissues other than the haematopoietic system. To achieve this, chapter 4 shows the fusion of the *GBA* gene to a protein transduction domain to investigate the potential for the cross-correction of untransduced cells by secreted GC protein.

The major morbidity found in type I GD, even among treated patients, is bone disease which we believe can be ameliorated by gene therapy of the HSC. To investigate this, in chapter 5 we will firstly establish whether genetic modification of HSC can lead to the differentiation of modified osteoclasts *in vitro* and *in vivo* and secondly investigate the occurrence of an osteoclast phenotype within the *GBA*^{-/-} mouse model (184).

2 Materials and Methods

2.1 Materials

Unless otherwise stated all reagents were supplied by Sigma-Aldrich, enzymes for cloning by Promega and cell culture reagents by Life Technologies.

2.1.1 Cloning

1kb Plus DNA ladder	Life Technologies
Q5 High-fidelity polymerase	New England Biolabs
Deoxynucleotides	Promega
XL-gold chemically competent E. coli	Agilent Technologies
QIAquick gel extraction kit	QIAgen
Glycerol	ACROS Organics

2.1.2 Plasmid preparation

QIAprep Spin Miniprep kit	QIAgen
QIAquick Maxiprep kit	QIAgen

2.1.3 Virus production

Centrifuge tubes	Beckman Coulter
------------------	-----------------

2.1.4 Virus titration

DNAeasy	Anachem
DNeasy blood & tissue kit	QIAGEN
Platinum® Quantitative PCR SuperMix-UDG with ROX	Life Technologies

2.1.5 Cell culture

MethoCult GF M3434	StemCell Technologies
Prostaglandin E ₂	VWR International
Macrophage colony stimulating factor (M-CSF)	R&D Systems
Receptor activator of nuclear factor kappa-B ligand (RANK-L)	R&D Systems
Dentine	Dr Derralynn Hughes (Royal Free Hospital, London)
TRAP staining kit	Sigma-Aldrich
Transwell inserts (diameter 12mm, pore size 0.4µm)	Fisher Scientific

2.1.6 Assays

Velaglucerase alfa (VPRIV)	Shire Human Gene Therapeutics
----------------------------	----------------------------------

FDGlu	Life Technologies
Citric acid	VWR International
Disodium hydrogen orthophosphate	VWR International

2.1.7 Western blotting

NP40	Calbiochem
Leupeptin	AppliChem
Aprotinin	AppliChem
NuPage gels	Life Technologies
MES buffer	Life Technologies
PVDF membrane	Millipore
Transfer buffer	Life Technologies
SeeBlue Plus2 pre-stained standard	Life Technologies
Pierce enhanced chemiluminescence western blotting substrate	Thermo Scientific

2.1.8 Immunocytochemistry

BSA	Promega
Rat serum	Dessi Malinova (Institute of Child Health)

PFA	Alfa Aesar
Mounting medium	Life Technologies
DAPI	Life Technologies
Phalloidin	Life Technologies
Slides	Thermo Scientific

2.1.9 Antibodies

Antibody	Manufacturer (catalogue number)
Mouse monoclonal to GC	Abcam (ab55080)
Mouse monoclonal to GAPDH	Santa Cruz Biotechnologies (sc-32233)
Rabbit polyclonal to GFP(FL)	Santa Cruz Biotechnology (sc-8334)
Rabbit polyclonal to BSA	Life Technologies (A11133)
Anti-mouse CD51-PE	eBioscience (12-0512)
Sheep anti-mouse, HRP conjugated	GE Healthcare (NXA931)
Donkey anti-rabbit, HRP conjugated	GE Healthcare (NA934V)

2.1.10 Solutions

Western blot lysis buffer	1% NP40, 10mM TRIS pH 8.0, 130mM NaCl, 1mM PMSF, 10mM NaF, 1mM DTT, 100mM Na ₃ VO ₄ 1% aprotinin, 200mM leupeptin
Western blot stripping buffer	15g glycine, 1g SDS, 10ml Tween20 in 1L water, pH 2.2
TAE (50x)	242g TRIS Base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA pH 8.0 in 1L distilled water
DNA loading dye	10% glycerol, 0.1% Orange G
Stopping solution	1M glycine in water, pH 10.4 with NaOH. Diluted to 0.25M in water for working solution
Mcllvaine citrate-phosphate buffer (MV 5.4)	0.1M citric acid, 0.2M disodium hydrogen orthophosphate. pH 5.2

2.2 Methods

2.2.1 PCR amplification

100ng template DNA was combined with 1 μ M of each primer, 200 μ M each dNTP, 1.25U Pfu polymerase, and 1X Pfu polymerase buffer and made up to a total volume of 50 μ l with sterile water. Initial denaturation was performed at 95°C for 2 minutes and followed by 30 amplification cycles consisting of denaturation at 95°C for 30 seconds, primer annealing at 58°C for 30 seconds (adjusted for each primer melting temperature) and extension at 72°C for 1 minute/kb, followed by a final extension step of 5 minutes at 72°C.

2.2.2 Restriction digests

1 μ g plasmid DNA was digested by 1U enzyme in 1x reaction buffer made up to a final volume of 20 μ l with sterile water. Digestion was performed at 37°C for 2 hours. If required, inactivation of the restriction enzyme was performed by heating the reaction mixture to the temperature and for the time given in the specification sheet.

2.2.3 Klenow polymerase treatment (filling 5'-protruding ends)

Digested DNA was incubated with 1U Klenow per microgram DNA in reaction mixture containing 50mM Tris-HCl, 10mM MgSO₄, 0.1mM DTT, 40 μ M each dNTP and 20 μ g/ml acetylated BSA for 10 minutes at room temperature. The reaction was terminated by heating to 75°C for 10 minutes.

2.2.4 Ligation

Insert DNA was ligated into 100ng digested pHR' plasmid backbone in a 3:1 or 5:1 ratio using 3U T4 DNA ligase in 1x reaction buffer overnight at room temperature

(approximately 22°C). The resulting ligated plasmid DNA was transformed into chemically competent XL-Gold E. coli and grown in the presence of ampicillin.

2.2.5 TOPO cloning

Digestion products were blunted with Klenow and ligated into the pCR- Blunt II-TOPO® subcloning vector using the Zero Blunt TOPO PCR cloning kit (Life Technologies) according to the manufacturer's instructions. Cultures were grown with kanamycin.

2.2.6 Transformation

2-4µl ligation mixture was gently mixed with 50µl chemically competent E. coli in a pre-cooled 14ml tube and incubated on ice for 30 minutes. Cells were heat-shocked at 42°C for 45 seconds and returned to ice for 2 minutes. 250µl SOC medium was added to the tube and incubated with shaking at 37°C for 1 hour and then plated on an LB-agar plate containing the relevant antibiotic (ampicillin for lentiviral plasmids, kanamycin for TOPO plasmids) at 50µg/ml. Plates were incubated at 37°C overnight.

2.2.7 Gel electrophoresis

DNA samples were added to 1x loading dye and applied to a 1% (w/v) agarose gel in TAE buffer alongside 10µl 1kb Plus DNA ladder and separated by electrophoresis at 80-120V in 1X TAE. DNA fragments were visualised by exposure to ultraviolet light using the UviDoc system.

2.2.8 Plasmid preparation

Bacterial clones were grown in 5ml (Miniprep yield = approximately 6µg) or 500ml (Maxiprep yield = approximately 500µg) LB broth with the relevant antibiotic at 50µg/ml overnight at 37°C with shaking. The following day cultures were pelleted by

centrifugation and plasmid DNA was extracted using QIAgen plasmid purification kits according to manufacturer's instructions.

2.2.9 Propagation of mammalian cell lines

All cells were incubated at 37°C with 5% CO₂. For long-term storage 1-5x10⁶ cells were pelleted and resuspended in 1ml freezing medium (90% FCS, 10% DMSO), transferred to cryovials and slowly cooled to -80°C in an isopropanol freezing box before being transferred to liquid nitrogen for long-term storage.

Human embryonic kidney 293T (HEK293T)

HEK293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% foetal calf serum (FCS) and 1% streptomycin/amphotericin (complete DMEM) until confluent and passaged using 1x trypsin-EDTA to remove cells from flask and diluted into new flasks.

Fibroblasts

Gaucher patient fibroblasts were obtained from Coriell Cell Repositories (GM00852) and cultured in Modified Eagle's Medium (MEM) with 10% FCS, 1% streptomycin/amphotericin and 2mM L-Glutamine. Passaging was performed using 1X trypsin-EDTA to remove cells which were seeded according to the cell data sheet.

Lymphoblastic cell lines (LCL)

Lymphoblastic cell lines (LCL) are suspension cell lines and were maintained in RPMI 1640 medium with 10 FCS and 1% penicillin/streptomycin.

2.2.10 Virus production

HEK293T cells were seeded at 1.5×10^7 cells/175cm² flask and allowed to reach 80-90% confluency. For each flask, 40µg vector construct, 10µg pMD.G2 and 30µg pCMV-ΔR8.74 plasmids were added to 5ml OptiMEM and filtered through 0.22µm filter. 10mM polyethylenimine was added to 5ml OptiMEM for each flask and passed through a 0.22µm filter. The two mixtures were combined and allowed to complex at room temperature for 20 minutes. Seeded cells were washed in OptiMEM to remove residual serum and 10ml transfection mixture was added to each flask and left at 37°C for 5 hours. After this time the medium was exchanged for 15ml DMEM and the flasks were returned to the incubator. Supernatant was harvested at 48 and 72 hours post-transfection, passed through 0.22µm filters and centrifuged at 98,000g for 2 hours. Pellets were resuspended in 150µl PBS and incubated on ice for 1 hour. The suspension was removed and centrifuged at 1,500g for 10 minutes and stored in 30µl aliquots at -80°C.

2.2.11 Virus titration

HEK293T cells were seeded in a 12 well plate at a density of 5×10^4 cells/well and left to adhere for 4 hours. Serial dilutions of virus in 1ml DMEM replaced the medium on the adhered cells which were incubated at 37°C for 72 hours. After this period, cells were harvested and titre was determined using two methods. For viruses containing a fluorescent protein (GFP), titre was calculated using flow cytometry performed on live cells. The titre of viruses without a fluorescent marker had to be performed using quantitative PCR as described below.

2.2.11.1 Flow cytometry

Cells were harvested with trypsin-EDTA and transferred to polystyrene flow cytometry tubes and pelleted by centrifugation at 1,500g for 5 minutes. Cells were washed twice in

PBS and finally resuspended in 500µl PBS and analysed for fluorescence by flow cytometry using the CyAn ADP cell analyser (Beckman Coulter, High Wycombe, UK).

Titre was calculated using the following formula:

$$\sum \frac{(\% \text{ positive cells}) \times (\text{dilution factor}) \times (\text{number of cells transduced})}{100}$$

2.2.11.2 Quantitative Real-Time PCR (qPCR)

72 hours post-transduction cells were washed, pelleted and DNA extraction was performed using 20µl DNAREleasey per sample according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using 100ng genomic DNA as template. The reaction mixture contained 0.9mM each primer, 0.2mM fluorescent probe and 1X qPCR SuperMix-UDG with Rox mastermix. The reaction was performed in triplicate with 40 cycles of: 95°C for 15 seconds and 60°C for 1 minute with quantification using an ABI Prism 7000 (Applied Biosystems, California, USA). Plasmid standards containing the human β-actin and HIV-1 WPRE sequences diluted in TE were kindly supplied by Dr Conrad Vink (UCL Institute of Child Health).

β-actin sequences

Forward primer: TGAGGATCTTCATGAGGTAGTCAG

Reverse primer: TCACCCACACTGTCCCATCTACGA

Probe: 5'FAM- ATGCCCTCCCCCATGCCATCCTGCGT-3'TAMRA

WPRE sequences

Forward primer: TGGATTCTGCGCGGGA

Reverse primer: GAAGGAAGGTCCGCTGGATT

Probe: 5' FAM-CTTCTGCTACGTCCCTTCGGCCCT-3' TAMRA

Virus titre was obtained using the following calculation:

$$\sum \left(\frac{\text{Qty WPRE}}{\text{Qty } \beta - \text{actin}} \right) \times \text{dilution factor} \times \text{number of cells transduced}$$

2.2.12 Osteoclast culture

Culled mice were sterilised with 70% ethanol. Bone marrow was harvested by removing the tissue from the long bones of the limbs and flushing with PBS using a 25 gauge needle. Extracted bone marrow was collected in a 15ml Falcon tube and pelleted by centrifuging at 1,500g for 5 minutes at room temperature. Samples were blinded to prevent experimenter bias. Cells were resuspended in 15ml S1MEM (OptiMEM with 10% FCS, 1% streptomycin/amphotericin, 2mM L-glutamine, 10^{-7} M PGE_2 and 86 μM M-CSF) and plated in a 75cm² flask and incubated at 37°C. 24 hours later 6mm dentine discs were sterilised by immersion in 100% ethanol, allowed to air-dry and soaked in OptiMEM + 10% FCS for 1 hour. Non-adherent cells were collected from flasks and pelleted at 1,500g for 5 minutes. Pellets were resuspended in S2MEM (OptiMEM with 10% FCS, 1% streptomycin/amphotericin, 2mM L-glutamine, 10^{-7} M PGE_2 , 38 μM M-CSF and 157 μM RANK-L) at a concentration of 5×10^6 cells/ml. 200 μl cell suspension was added to sterilised 6mm dentine discs or 13mm glass coverslips in a 96-well plate (6-8 discs/mouse) and incubated overnight at 37°C and 5% CO_2 . The following day discs were transferred to 6-well plates with 4 discs/well in 3ml S2MEM. A half media change was performed after 48 hours. On day 7, cultures were acidified by complete media change with acidified S2MEM (82 μl concentrated HCl in 100ml S2MEM). Cultures were

terminated 48 hours after acidification; discs were washed in PBS and fixed in 2.5% citrate-acetate buffer for 30 seconds and washed in distilled water.

2.2.12.1 Assessment of osteoclast activity by resorption

Cells on dentine discs were removed using ProteoJet lysis solution (Fermentas). Discs were washed in distilled water and incubated in 1% toluidine blue for 30 seconds and washed in distilled water to remove residual dye. Areas of bone resorption were counted using a Nikon Eclipse 400 microscope.

2.2.12.2 Identification of osteoclast-like cells by TRAP staining

Cells cultured on glass coverslips were terminated as above and stained for tartrate resistant acid phosphatase (TRAP) using the leukocyte acid phosphatase detection kit from Sigma-Aldrich according to the manufacturer's instructions.

2.2.13 Bone marrow cultures

Culled mice were sterilised with 70% ethanol. Bone marrow was harvested by removing the tissue from the long bones of the limbs and flushing with PBS using a 25 gauge needle. Marrow was collected in a 15ml Falcon tube and pelleted by centrifugation at 1,500g for 5 minutes at room temperature. Lineage negative cells (representing the murine haematopoietic stem cell component) were isolated using the MACS Lineage Cell Depletion Kit (Miltenyi Biotec) according to the manufacturer's instructions. Isolated cells were seeded at a density of 10^6 cells/ml and transduced with lentivirus overnight. The following day cells were seeded in MethoCult medium (M3434, StemCell Technologies) for colony forming cell assays according to the manufacturer's protocol.

2.2.14 Assays

2.2.14.1 Glucocerebrosidase

Two assays were used to establish glucocerebrosidase activity levels. Both used non-fluorescent substrates but followed different protocols.

2.2.14.1.1 FDGlu assay

Cells were diluted to 10^5 - 10^6 cells/ml and 100 μ l added to each of two FACS tubes. 10 μ l 10mM conduritol- β -epoxide (CBE) was added to one tube and 10 μ l DMEM to the other. Cells were incubated at room temperature for 1 hour. 100 μ l 2mM fluorescein- β -D-glucopyranoside (FDGlu) was added to each tube and mixed gently then incubated at 37°C for 1 minute. 790 μ l DMEM was added to both tubes and incubated in the dark at room temperature for 45 minutes. Cells were centrifuged at 1,500g for 5 minutes and resuspended in PBS twice. Cells were analysed using flow cytometry using the CyAn ADP cell analyser with fluorescence read at excitation 490 nm, emission 515 nm.

2.2.14.1.2 4MUG

Cell pellets were resuspended in 10 μ l distilled water and lysed by ultrasonication for 10 seconds at amplitude 6 using an MSE 100W sonicator. Supernatant samples were centrifuged at 16,000 x g to pellet cell debris and transferred to new tubes. Samples were diluted to a protein concentration of 1mg/ml in distilled water. 10 μ g protein sample was added to 25 μ l MV 5.4 with 22.35mM sodium taurocholate and 5mM 4-methylumbelliferyl- β -D-glucopyranoside was added to each tube and mixed. Samples were incubated at 37°C for 1 hour then 1100 μ l stopping solution (0.25M glycine buffer pH 10.4) was added to terminate the reaction. Fluorescence was read at excitation 365 nm, emission 450 nm using the Perkin Elmer LS 55 fluorometer (Perkin Elmer,

Massachusetts, USA). 1nmol 4-methylumbelliferone was used as a standard. Enzyme activity was calculated as nmol/hr/mg protein.

2.2.14.2 Chitotriosidase

Supernatants were harvested and centrifuged for 5 minutes at 16,000 x g to pellet cell debris. On ice 5µl supernatant was added to 21µM 4-methylumbelliferyl-β-D-N,N',N''-triacetylchitotriose and mixed. Tubes were incubated for 60 minutes at 37°C then at timed intervals 1000µl of 0.25M glycine buffer (pH 10.4) was added to each tube to terminate the reaction. Fluorescence was read at excitation 365nm, emission 450nm using the Perkin Elmer LS 55 fluorometer. 16µM 4-methylumbelliferone was used as a standard. Activity was calculated as nmol/hr/ml supernatant.

2.2.15 Western blotting

2.2.15.1 Preparation of cell lysates

10⁶ cells were pelleted by centrifugation and resuspended in 200µl lysis buffer and incubated on ice for 5 minutes. Debris was pelleted by centrifugation at 16,000g for 15 minutes at 4°C. 200µl Laemmli buffer was added to the supernatant and samples were heated to 85°C for 5 minutes. After this samples were kept at 4°C or stored at -20°C.

2.2.15.2 Gel electrophoresis

30µl sample was run on a NuPage gel in 1X MES SDS buffer at 150V for 1 hour alongside 10µl SeeBlue Plus 2 prestained protein standard.

2.2.15.3 Membrane transfer

Protein bands were transferred to a PVDF membrane using 2X transfer buffer at 18V in a BioRad semi-dry transfer machine. After transfer membranes were blocked in 4% milk to minimise non-specific staining on the roller for 1 hour at room temperature.

2.2.15.4 Staining and visualisation

Primary antibodies were added at a dilution of 1:250 or 1:500 in 3ml 4% milk and incubated overnight with rolling at room temperature. Antibody mixture was removed and blot washed with 3x3ml PBS with 0.05% Tween-20 (PBST) for 5 minutes. Secondary antibodies were added at a dilution of 1:1000 in 3ml 4% milk and incubated for 45-60 minutes with rolling. Antibody mixture was removed and blot washed with 3x3ml PBST for 5 minutes before blots were developed with ECL. ECL reagents were mixed in a 1:1 ratio to a volume of 1ml. Mixture was applied to the blot and incubated at room temperature for 5 minutes after which time excess ECL mixture was removed. Bands were then visualised using the UVIchemi chemiluminescence detection system (UVItec, Cambridge, UK).

Where necessary, visualised blots were stripped of antibodies by incubation with two applications of 5ml stripping buffer, incubated for 10 minutes at room temperature with shaking, followed by three washes in 3ml PBST for 10 minutes to remove residual stripping buffer. Blocking and visualisation of alternative protein bands could then be performed as described above.

2.2.16 Immunocytochemistry

Cells were seeded on 13mm coverslips 4 hours before staining. Cells were washed in PBS and fixed in 4% paraformaldehyde for 20 minutes at room temperature, permeabilised in 0.1-0.5% Triton and blocked in 2% rat serum. Primary antibodies were diluted in PBS with 0.2% BSA and incubated with cells for 1 hour. Secondary antibodies and cell markers were also diluted in PBS with 0.2% BSA and were incubated with cells for 45 minutes to an hour in the dark. Coverslips were mounted using ProLong Gold and stored in the dark before imaging. Between each step coverslips were washed twice in PBS with 0.2% BSA.

Confocal images were taken using a Zeiss LSM 710 inverted confocal microscope (Leica, Milton Keynes, UK).

2.2.17 Software

Microscopy and electrophoresis gel images were processed with ImageJ (NIH, Bethesda, Maryland). Statistical testing was performed using GraphPad Prism 5 (San Diego, California).

3 Vector production and characterisation

3.1 Aims

- To produce and characterise a lentiviral vector carrying the glucocerebrosidase gene
- To show the vector produces full length, active glucocerebrosidase protein
- To correct enzyme deficiency of patient derived cells using this vector
- To investigate the biomarker chitotriosidase as a read out of correction efficiency of fibroblasts *in vitro*

3.2 Introduction

Gaucher disease (GD) arises from mutations in the glucocerebrosidase (*GBA*) gene leading to a deficiency in the enzyme glucocerebrosidase (GC) (3). The majority of affected cells are part of the haematopoietic system (primarily the macrophage) and it has been shown that allogeneic haematopoietic stem cell transplant (HSCT) from a healthy donor can be curative (71, 98-101). However due to a shortage of suitable donors and the high risk of mortality associated with HSCT from an unrelated donor, it is not performed routinely for the treatment of GD (98, 102).

Regardless of its current usage, the curative effect of HSCT indicates that correction of the haematopoietic system is sufficient to alleviate disease; a hypothesis which is further supported by the response of patients to the current, macrophage targeted, enzyme replacement therapy (ERT) (109, 123, 124). It is this evidence that has given rise to the hypothesis that *ex vivo* gene therapy of HSCs could be used to treat, and potentially cure, type I GD.

Gene therapy could be preferable to HSCT as it can use autologous stem cells which reduces the risk of graft versus host disease and other complications of unrelated donor HSCT (381). In addition, correcting stem cells at the genetic level potentially allows for a permanent curative effect as enzyme will be produced throughout the lifetime of the cells, dramatically reducing the dependence of patients on ERT as shown in the ADA-SCID clinical trial (226-228).

Before the advent of ERT, a number of groups were investigating the possibility of developing gene therapy for GD but the licensing of Ceredase® in 1991 (109) caused researchers to suspend this work. However, given the limited impact of ERT on skeletal symptoms (50, 153, 157) and the risk of symptom recurrence if supply is interrupted (146, 147) we believe that the time to reconsider gene therapy has come and so this project aims to produce and test lentiviral vectors which have the potential for use in treating GD.

The following chapter will describe the design of the *GBA* vector produced in this project; it will also detail the efforts made to characterise the protein produced from integrated vector, and the ability of the virus to correct patient material.

3.3 Results

3.3.1 Patient interest in gene therapy for type I Gaucher disease

Gene therapy for GD was first investigated in the late 20th century but efforts to develop it were abandoned for several reasons. Firstly, serious adverse reactions were reported in a number of participants in gene therapy trials for immunodeficiencies which cast doubt on the suitability of the procedure and the vectors in use (234-236). At the same time, no response was found in a human trial of gene therapy for GD (373). These results coupled with the approval of the enzyme replacement therapy manufactured by Genzyme (109, 124, 156) contributed to the discontinuation of the majority of gene therapy investigations.

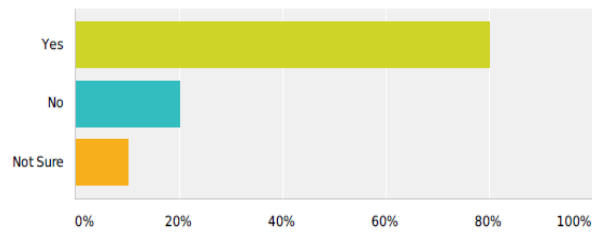
This has produced a lasting belief amongst clinicians that gene therapy is of no interest to the Gaucher community. To address this concern, a small survey of type I patients and their families was conducted with the assistance of the Gauchers Association to gauge the levels of patient interest. A factsheet (Appendix 7.3) explaining the rationale behind gene therapy and the procedure involved was provided to participants with a follow-up questionnaire to assess their response (Appendix 7.4).

The results showed that 80% respondents would consider gene therapy as a viable option for GD and 50% might prefer a fully licenced gene therapy treatment to ERT (Figure 3.1B). Of those who answered not sure (20%) or no (30%) to question 6, the main comments were concern about the possible side effects (especially those connected with the conditioning regimen) and patients with mild/stable disease expressing that they would be happier to continue with ERT (Appendix 7.5).

A)

Q4 After reading this fact sheet would you consider gene therapy as an option for Gaucher disease?

Answered: 10 Skipped: 0

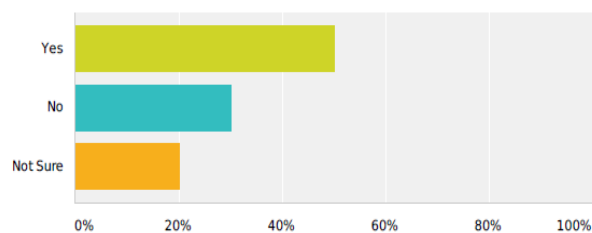


Answer Choices	Responses
Yes	80% 8
No	20% 2
Not Sure	10% 1
Total Respondents: 10	

B)

Q6 If gene therapy was offered to you as a fully approved treatment option (once it has passed through trial stages) for you or your child, do you think it would be a preferable option to ERT?

Answered: 10 Skipped: 0



Answer Choices	Responses
Yes	50% 5
No	30% 3
Not Sure	20% 2
Total Respondents: 10	

Figure 3.1 Patient response to gene therapy for GD questionnaire

A small group of type I GD patients were given a factsheet describing how gene therapy could be used to treat GD and were then asked for their response to the proposal. Answers to two of the questions are shown here and show that 50% of these patients would welcome gene therapy as an option for GD with another 20% remaining undecided. Patients were able to select more than one response for each question.

3.3.2 Vector production

The first aim of this project was to create a lentiviral vector carrying the *GBA* gene which could be used for gene therapy.

The lentiviral backbone consists of a minimal viral genome containing the central polypurine tract (cPPT), a packaging signal (Ψ) and the woodchuck post-transcriptional regulatory element (WPRE) flanked by long terminal repeats (LTR) of which the 3' LTR is self-inactivating. In addition to these elements, the backbone used in this project contains the spleen focus-forming virus promoter to drive expression of the transgene cassette.

Previous unpublished work by Dr Ahad Rahim (UCL Institute for Women's Health) inserted the genes encoding glucocerebrosidase (GC) and enhanced green fluorescent protein (eGFP) separated by an internal ribosomal entry site (IRES) into the lentiviral backbone (Figure 3.2A). The IRES element allows two genes to be transcribed from the same mRNA independently of each other so that the result is not a fusion protein (382). Concurrent production of a marker protein such as eGFP is often used in order to confirm transduction of cells and that introduced DNA is being expressed. However there have been recent studies which suggest that eGFP is incorrectly polyubiquitinated and therefore may not be efficiently degraded by the cell. It has been suggested that the protein accumulates in the lysosome where it may interfere with enzymatic function (383). As GC is a lysosomal protein it was decided to remove the *eGFP* gene from the plasmid, kindly donated by Dr Rahim, to produce a lentiviral vector containing only the *GBA* gene (Figure 3.2B). Additionally, the ultimate goal of this work is to produce a product which can be translated to the clinic and must therefore be free of any non-human sequences, including *eGFP*.

Removal of the *eGFP* sequence was achieved by simultaneously digesting the existing vector with the restriction enzymes XhoI and MluI to excise the IRES-*eGFP* fragment. The ends of the two resulting fragments were filled in using DNA polymerase I large fragment (Klenow) and the larger of the two sequences, which corresponded to the lentiviral backbone containing the SFFV promoter, *GBA* gene and WPRE sequence, was purified by agarose gel electrophoresis and religated using T4 DNA ligase. This plasmid was transformed into XL Gold chemically competent cells and grown on LB-agar plates containing ampicillin in order to identify correct clones. Removal of the IRES-*eGFP* sequence was confirmed by digestion with the restriction enzyme HindIII (Figure 3.2C) and by sequencing (Appendix 7.2).

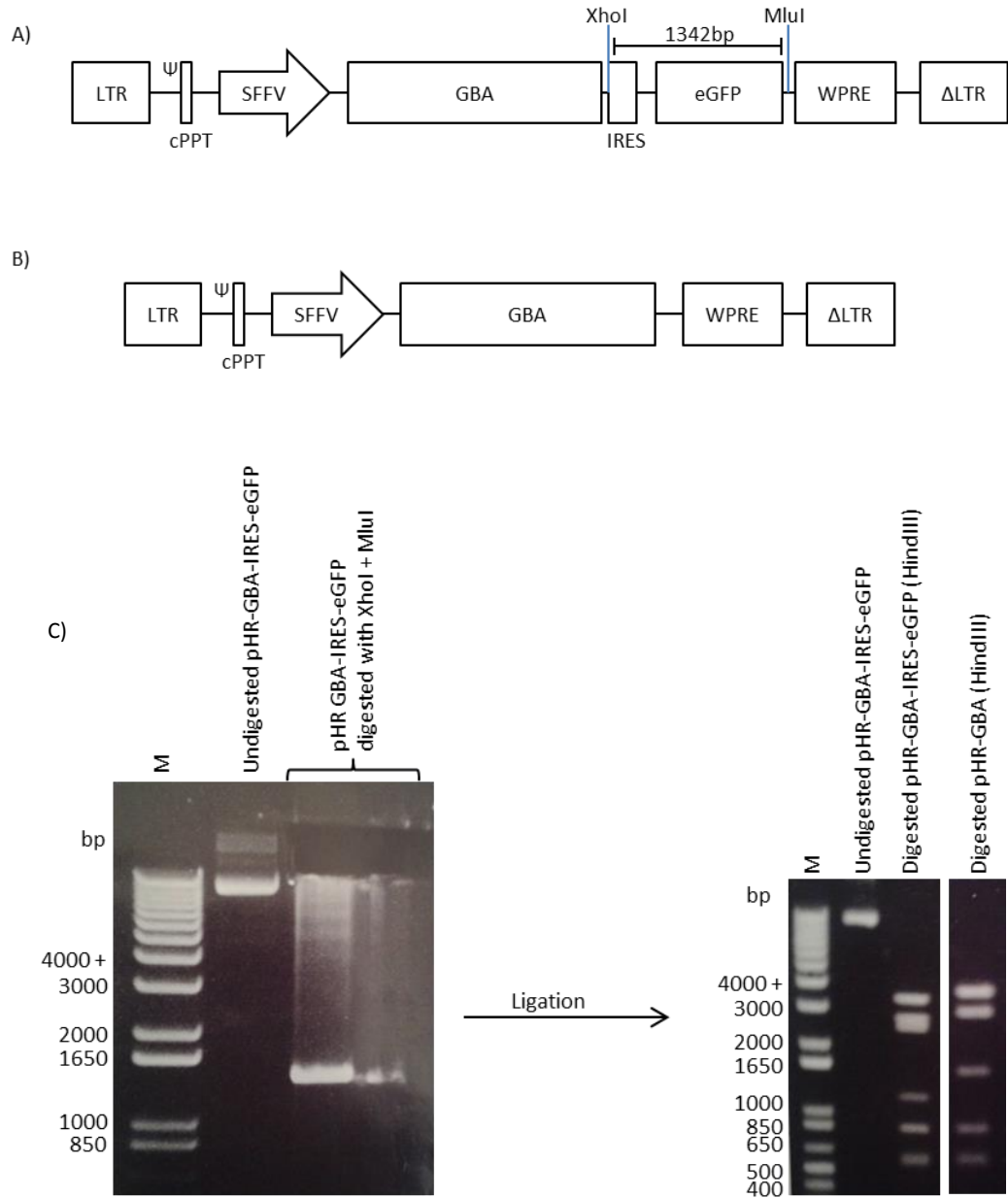


Figure 3.2 Removal of IRES-eGFP to give the pHR-GBA plasmid

A) LNT-GBA-eGFP plasmid produced by Dr Ahad Rahim and donated for use in this study. Vertical lines show recognition sites for restriction endonucleases. B) LNT plasmid carrying only the *GBA* gene produced by digestion and relegation of the plasmid in A). LTR: long terminal repeat, Ψ: packaging signal, cPPT: central polypurine tract, SFFV: spleen focus forming virus LTR/promoter, *GBA*: glucocerebrosidase, *IRES*: internal-ribosomal entry site, *eGFP*: enhanced green fluorescent protein, *WPRE*: woodchuck

hepatitis post-transcriptional regulatory element Δ LTR: self-inactivating LTR. C) electrophoresis gels showing the excision of the IRES-*eGFP* fragment (1342bp) from the pHR-GBA-IRES-eGFP plasmid. Subsequent blunting and ligation of the purified fragment (16871bp) gave the pHR-GBA plasmid. Excision of the IRES-eGFP fragment was confirmed by digestion with HindIII and sequencing.

3.3.3 Glucocerebrosidase activity can be determined with fluorometric assays

A common method of detecting enzyme activity is to use a substrate which emits a fluorescent signal after enzymatic cleavage. There are multiple substrates available for the detection of GC activity and two of them were tested in this project – fluorescein- β -D-glucopyranoside (FDGlu) and 4-methylumbelliferyl- β -D-glucopyranoside (4MUG). The structures of both of these substrates are shown in Figure 3.3A, along with the sites of cleavage by GC (dashed lines). The protocol for both of these assays has been described elsewhere with the chief difference being that the FDGlu assay is performed on live cells and measured by flow cytometry (384) whereas the 4MUG assay uses cell lysates with fluorescence detected by spectrophotometry (385, 386).

As readout is measured by flow cytometry, the FDGlu assay gives results as a percentage of cells expressing the enzyme but is unable to give information on the levels of actual enzyme activity within cells. This makes a useful tool for detecting successful transduction of target cells but more information is required to determine the extent of enzyme activity and whether phenotypic correction has occurred. It is also worth noting that the FDGlu assay gives highly variable results as shown in Figure 3.3B where the assay was run on a single population of HEK293T cells at different time points.

By contrast the 4MUG assay is performed on lysed cells giving readout in terms of enzyme activity which is more informative as it allows for the detection of true molecular correction. The assay is also sensitive enough to distinguish the different activity levels in wild type, heterozygote and *GBA* knockout mice (Figure 3.3C), something which is otherwise only possible by sequencing. Because of the greater applicability of the 4MUG assay and the fact that its readout allows for a direct measurement of correction, it was decided that further experiments would be analysed using the 4MUG assay only.

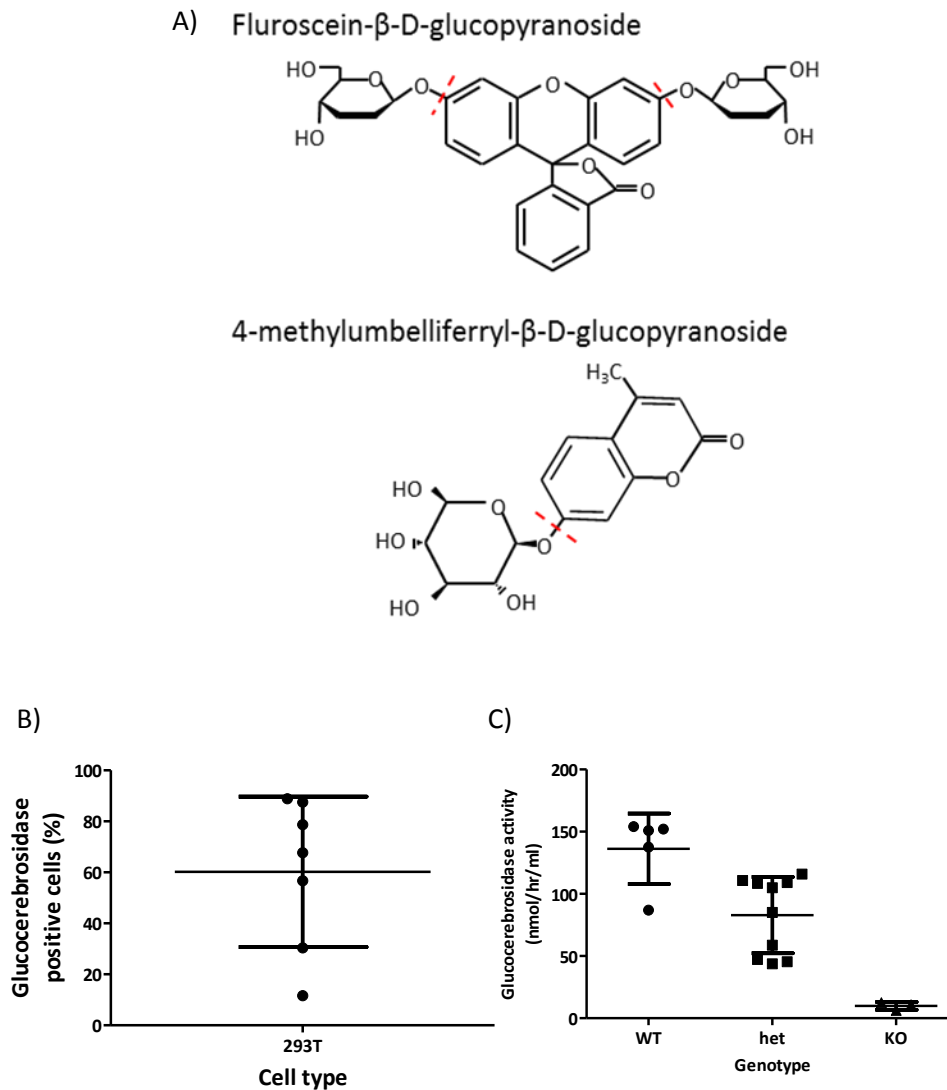


Figure 3.3 Fluorescence assays for the detection of glucocerebrosidase activity

A) Substrates used in the detection of GC activity, both are non-fluorescent before cleavage by the GC enzyme. B) HEK293T cells assayed with the substrate FDGlu. Large error bars showing standard deviation indicate the high variability of results from this assay; each point indicates a replicate from the same population. C) Macerated livers of 2-week old mice from the *GBA*^{-/-} colony were assayed using 4MUG and it was possible to determine phenotypic status (confirmed by genotyping performed by Dr Ahad Rahim). Each point represents an individual mouse.

3.3.4 Vector constructs produce functional glucocerebrosidase protein

The vector produced here contains the full length *GBA* sequence with no mutations or frame-shifts as was confirmed by sequencing (Appendix 7.2).

To test the functionality of the encoded protein, lentivirus made from the construct was used to transduce HEK293T cells at increasing multiplicity of infection (MOI). After a week in culture, cells were harvested and enzymatic assays and western blots were performed to check for an increase in the levels and activity of GC protein relative to untransduced cells.

Below an MOI of 50 there was very minimal increase of protein expression or activity but at higher MOI, a significant increase in protein expression and enzyme activity was found (Figure 3.4). The western blot shown in Figure 3.4A shows that protein levels appear to double when MOI is increased from ten to 100, this is a smaller increase than might be expected with such a large increase of MOI and is also reflected in the modest increase of protein activity between the two viral concentrations (Figure 3.4B). Although the GAPDH band for MOI 10 sample appears to be smaller than that for the other two concentrations, it is likely that this is due to incomplete visualisation of the band rather than an error in sample loading. The limited increase in protein expression and activity between MOI 10 and 100 could be due to high levels of transduction resulting in significant overexpression of the protein leading to secretion. It is known that *GBA* contains a secretion signal and it is our hypothesis that accumulated protein would be secreted using this endogenous sequence. This hypothesis will be addressed in the following chapter but would account for the small increase in intracellular GC between the two conditions especially as HEK293T cells are known to express sufficient *GBA* as is indicated by the GC activity levels in the MOI 0 condition.

With the higher virus concentrations in the western blot shown in Figure 3.4A it appears that there are multiple bands being detected with the GC antibody (upper panel). This was unexpected as GC western blots should give only one band at a molecular weight of 60kDa however it was also found on other western blots presented in this thesis including a western blot performed on the velaglucerase alfa ERT preparation (Figure 4.6) and is most notable where there are high concentrations of GC. One possible explanation for this phenomenon is that the bands of lower molecular weight correspond to glycosylation variants of the enzyme, something which could be established using mass spectrometry. As part of this project we attempted to look at the glycosylation state of the enzyme produced by mass spectrometry but unfortunately the protein produced was not pure enough for the investigation to succeed and so no data is shown.

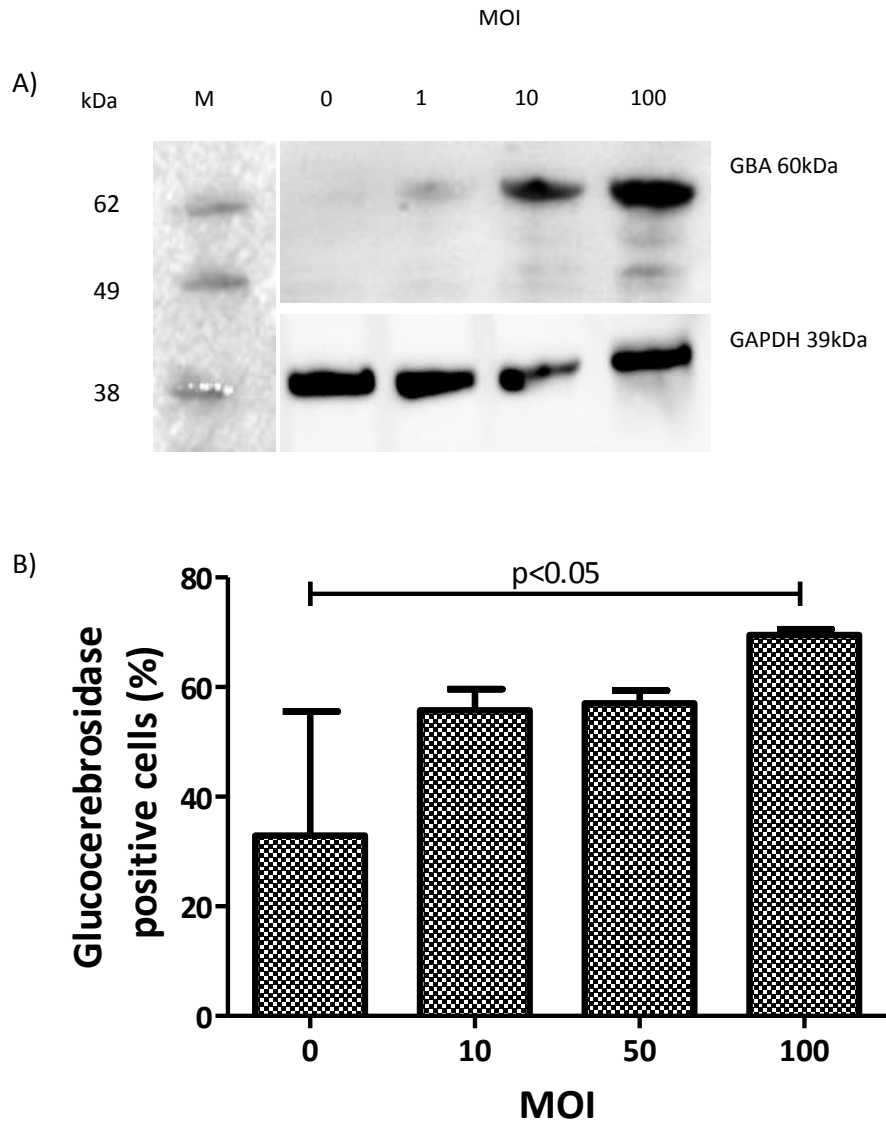


Figure 3.4 Glucocerebrosidase expression and activity in HEK293T cells

HEK293T cells were transduced with increasing levels of *GBA* vector and harvested after 7 days to assess levels of GC expression and activity. A) Western blot showing increase of GC protein in transduced cells. Lower bands correspond to GAPDH which is used as an internal control for protein loading. B) FDGlu assay showing that transduction of HEK293T cells results in increased GC activity within the live cells although significance was only achieved when an MOI of 100 was used. n=3, significance tested using one-way ANOVA with Dunnett's post-hoc test. Error bars show standard deviation.

3.3.5 Enzyme activity levels peak at day 3 post transduction

A time course experiment was run over 14 days to investigate the effect of time on enzyme expression post-transduction. This data is important for optimisation of protocols as it will inform the choice of time points for subsequent experiments.

HEK293T cells were transduced with virus carrying the *GBA* gene at an MOI of 50 and samples were harvested daily for 14 days. Cell viability was assessed at each time point to ensure that there was no toxicity of vector or protein. At the end of the time course cell pellets were lysed and investigated using a combination of western blotting to look for protein of the correct size and enzymatic assay to assess enzyme activity levels.

After transduction, viability levels fell slightly as is expected when cells have to cope with a viral infection. Upon infection host cell machinery is used to transcribe and translate the viral genome which places a pressure on the cell which can lead to cell death and is one reason that levels of viral transduction have to be carefully controlled to prevent high levels of cell mortality. The drop in viability was not significant when compared to cells transduced with a control (eGFP) vector showing that the transgene in the experimental vector is no more toxic to transduced cells than the control protein. Viability levels had recovered by day 14 and remained above 80% throughout the experiment showing that the effect on cell survival is only transient (Figure 3.5A).

On the second day post-transduction, enzyme activity levels exceeded the level deemed healthy for human fibroblasts which was an increase of 10-fold relative to levels on day zero. At their peak (day three) enzyme activity levels were 15 times greater than pre-transduction. Activity levels fell slightly post-day three which is most likely due to the clearance of non-integrated viral genomes and RNA which had not been reverse transcribed, but stabilised at a level within the unaffected range (Derek Burke, personal communication) and approximately 13 times greater than that of untransduced cells

(Figure 3.5B). In order to confirm that the reduction in enzyme activity seen post-day 3 is due to clearance of unintegrated genomes, it would be necessary to perform a qPCR investigation to assess the number of genomes in the cell populations.

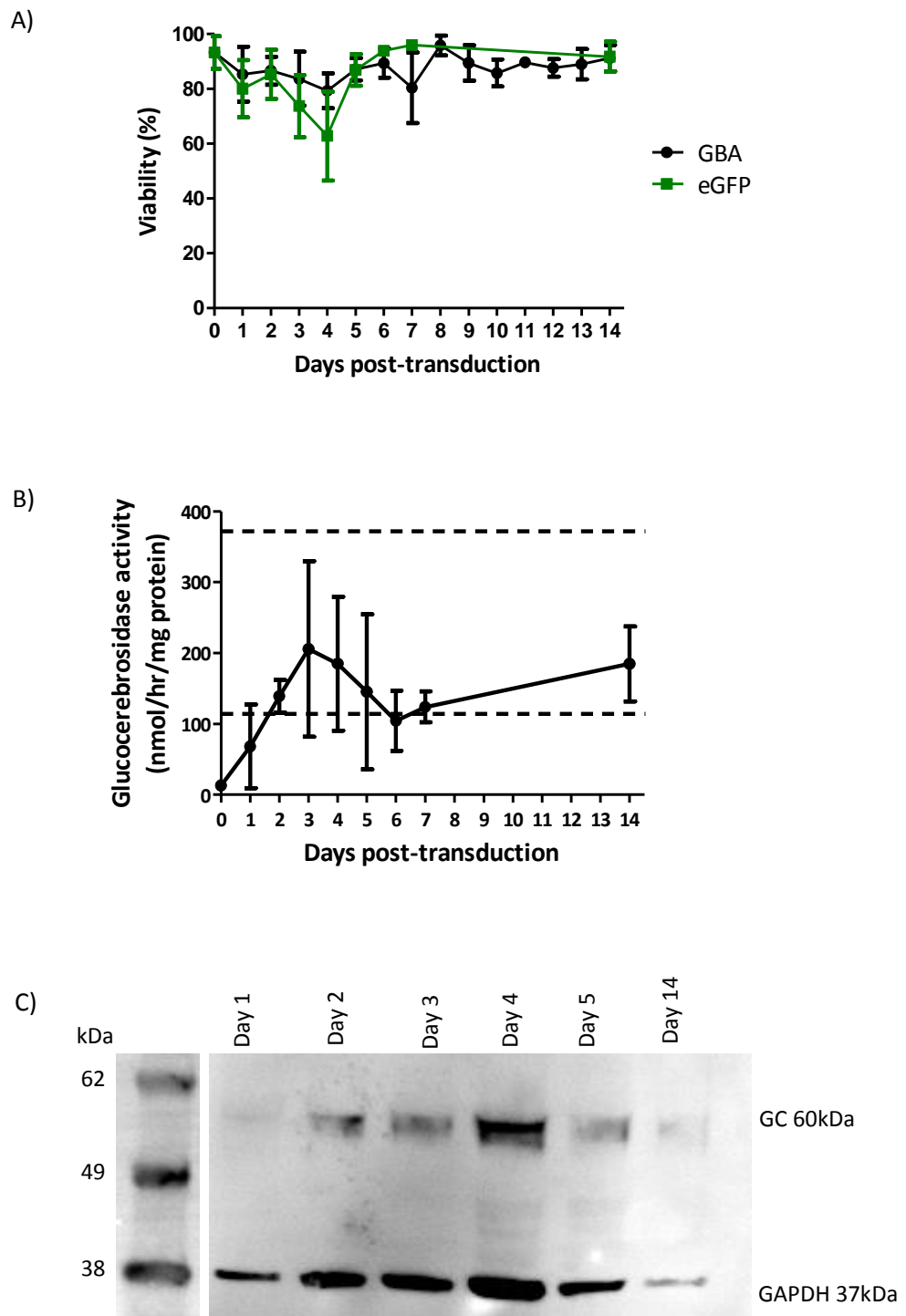


Figure 3.5 Glucocerebrosidase expression and viability over time in transduced cells.

HEK293T cells were transduced with lentivirus carrying either eGFP or *GBA* at an MOI of 50 and viability was assessed every day over a 14 day period for viability and GC activity.

A) Viability was assessed using trypan blue exclusion dye. No significant change was found at any day in either condition and viability remained above 60% throughout. B) Levels of GC activity after transduction. Dashed lines indicate the unaffected range for fibroblasts. C) A representative western blot showing increase of protein between days one and four with a slight fall thereafter. All error bars show standard deviation.

3.3.6 Integrated vector can restore enzyme activity in patient

fibroblasts

Fibroblasts derived from a type I patient who was a compound heterozygote with the genotype N370S/84GG and was reported to have 6% normal GC activity were purchased from the Coriell Institute (New Jersey, USA) to test the vector. Although fibroblasts are not of haematopoietic lineage and are not the primary disease cell in GD, this cell population was used as the only commercially available patient cell types are fibroblasts and B-lymphocytes. Of the two, fibroblasts express a higher level of GC and so are better suited to this project.

The fibroblasts were transduced at an MOI of 50 to confirm that the vector delivered gene is capable of correcting the inherent metabolic defect which causes GD. One month post-transduction a western blot was performed on fibroblasts and the 4MUG assay was run to check for restoration of protein expression and activity respectively (Figure 3.6). The time period between transduction and harvesting cells for the assays was longer than would be expected with other cell lines because these fibroblasts were very slow growing. Enzyme activity was significantly increased to a value which exceeds the unaffected range and which is 25 times greater than the baseline, untransduced activity levels. No increase in enzyme activity was seen when the same cells were transduced with a control vector expressing a fluorescent protein in place of the *GBA* gene confirming that the increase in enzyme levels is a specific effect of the *GBA* vector and that viral transduction does not have an inherent effect on enzyme activity levels.

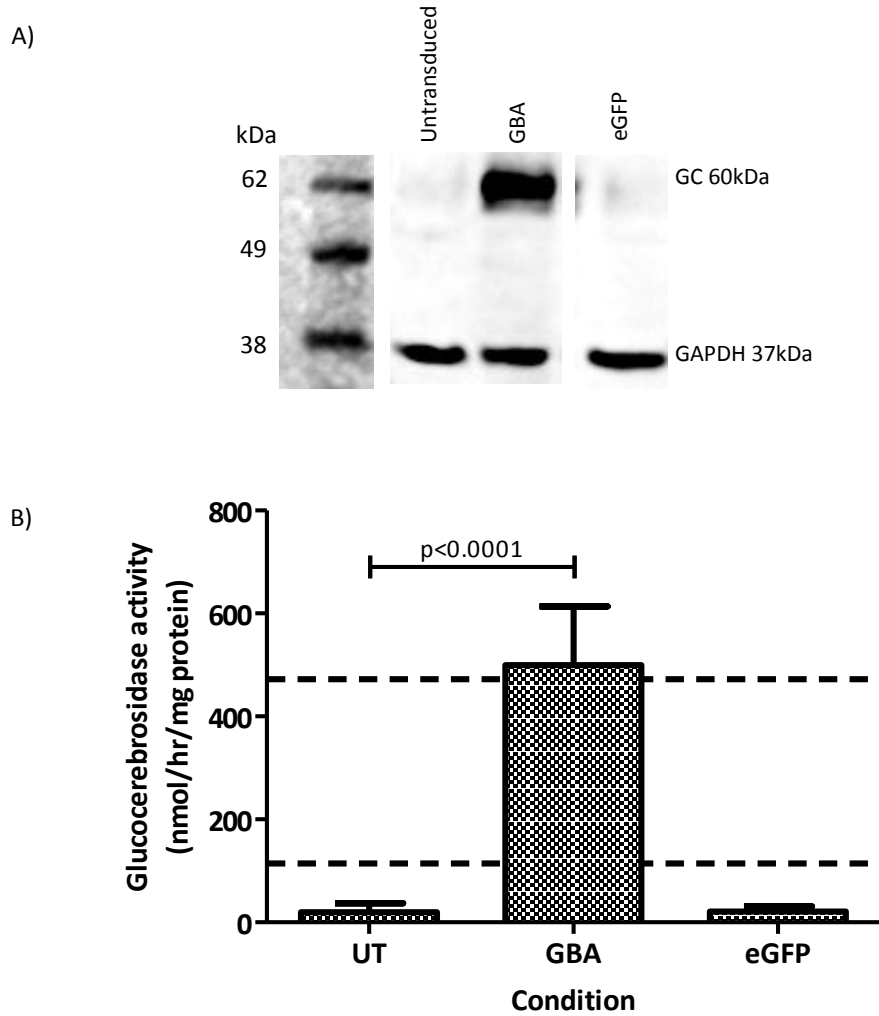


Figure 3.6 Expression and activity of glucocerebrosidase is restored in patient fibroblasts after transduction with LNT-GBA

Fibroblasts derived from a type I GD patient were transduced with a lentiviral vector carrying either the *GBA* or *eGFP* gene and grown for one month to give enough cells to assay. A) Western blot on transduced cells showed increase in the amount of expressed GC protein. B) Enzyme activity assay on the same cells reveals restoration of healthy levels of GC activity (dashed lines) when transduced with the *GBA* vector but not with *eGFP* vectors. Error bars show standard deviation, $n=7$, significance was tested with one-way ANOVA with Dunnett's post-hoc test.

3.3.7 Chitotriosidase activity cannot be measured in fibroblasts *in vitro*

Chitotriosidase (ChT) is an immune protein which is secreted into plasma and is elevated in both treated and untreated GD patients. It is an important biomarker for GD as ChT levels fall in response to treatment (179).

Personal communication from Dr Filippo Vairo (Hospital de Clínicas de Porto Alegre, Brazil) suggested that, although fibroblasts do not produce significant amounts of ChT, it is possible to observe changes in expression levels *in vitro* which could be a useful marker of correction in the patient-derived fibroblasts used in this study.

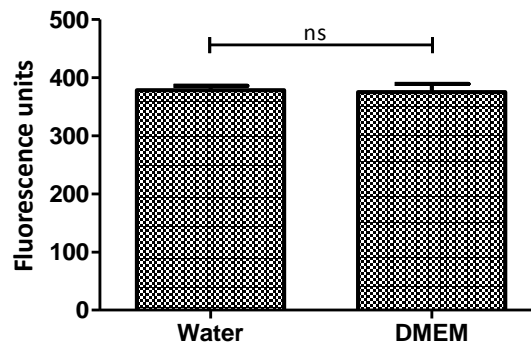
Like GC, ChT can be measured using a non-fluorescent substrate which emits a fluorescent signal after cleavage by the enzyme. Because this assay is performed on the supernatant of cultured cells, it was first necessary to assess the culture medium for background fluorescence levels. The results, shown in Figure 3.7A, reveal that there is no significant difference in fluorescence levels when the fluorescent standard 4-methylumbelliferone is diluted in water compared to the DMEM culture medium. This indicates that the medium neither autofluoresces nor quenches the signal from the fluorescent product and so would not interfere with the study measurements.

GD and unaffected fibroblasts were cultured in DMEM and samples were removed from the medium after 6 days to test for ChT activity (Figure 3.7B). The results showed that there was no significant difference in the enzyme activity levels after transduction with the GBA vector suggesting that ChT is unaffected in fibroblasts and therefore cannot be used as a surrogate measurement of correction in this work.

This result was not entirely unexpected as the majority of ChT is expressed from macrophages and other cells of the immune system whereas fibroblasts have no immune

function. Although it appears that there may be a slight reduction in ChT in the patient condition compared to the unaffected cells, the difference is very slight compared to that seen in plasma where ChT levels of untreated patients are on average 600 times greater than those of unaffected individuals.

A)



B)

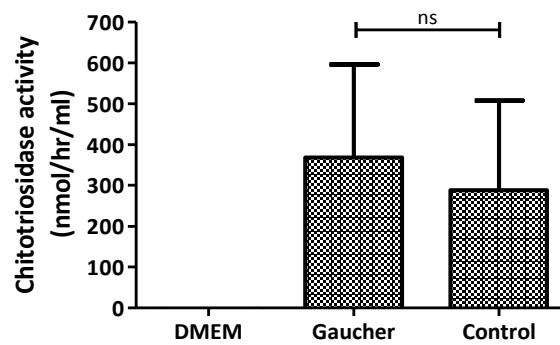


Figure 3.7 Chitotriosidase cannot be measured in cultured fibroblasts.

A) Diluting 4-methylumbelliferone standard in DMEM does not quench or increase fluorescence levels compared to diluting in water. B) Chitotriosidase activity measured in the supernatant of control and Gaucher fibroblast cultures reveals no significant difference between conditions. Error bars show large standard deviation due to variability of the assay but an unpaired t-test gave a p value of greater than 0.5 indicating no significant difference.

3.4 Summary

In this chapter a lentiviral construct has been produced which contains a wild type copy of the glucocerebrosidase gene free from any reporter sequences. Testing of this construct in HEK293T cells revealed that transduction with virus bearing this construct results in expression of protein of the correct molecular weight with enzymatic activity. Applying the virus to patient-derived fibroblasts corrects the enzymatic defect seen in these cells and restores enzyme activity levels to within the healthy range.

A time course experiment to look at the changes in enzyme activity post-transduction showed that the levels of activity peak at around day three 3 and then fall slightly over the next four days but become stable at a level ten times greater than baseline. This is the expected pattern as when it is first introduced into the host cell the viral RNA genome will be expressed almost immediately producing a spike in protein levels. Not all copies of the viral genome will integrate and any copies which do not will be cleared from the cell and will not contribute to long term enzyme activity levels therefore, protein produced after the first few days is the result of integrated gene expression.

One of the problems encountered in this chapter was the method of measuring GC activity. The original assay used (FDGlu) was measured by flow cytometry and so was unable to give a quantifiable measurement of intracellular enzyme activity although it could indicate levels of transduction. It also gave incredibly variable results even when the same cell population was being assayed (Figure 3.3B). It was therefore decided to change to a different assay (4MUG) which was more reliable and gave readout in terms of exact enzyme activity. Both assays use similar substrates which release fluorescence when cleaved by GC but the method of fluorescence detection is different and performing the 4MUG assay on cell lysates makes it more useful to this work than the whole cell measurements generated by the FDGlu assay.

In an attempt to develop another measure of cell correction, the use of the biomarker chitotriosidase (ChT) was assessed as it was not possible to use substrate storage reduction as a readout of cellular correction. This is because macrophages are the only cells to display accumulation of substrate and this work was performed using fibroblasts. ChT was chosen because it is well established as a biomarker for the monitoring of Gaucher disease in patients (151, 179) and personal communication from a GD specialist suggested that it is possible to measure ChT in fibroblast cultures. However this investigation showed that this is not the case as there was no significant difference in ChT levels between Gaucher disease and non-GD fibroblast populations and therefore it was only possible to use enzyme activity levels to indicate correction.

4 Secreted glucocerebrosidase for the cross-correction of untransduced cells

4.1 Aims

- To add wild type and modified HIV-1 TAT protein transduction domains (PTD) to the *GBA* cDNA
- To show that *GBA* is secreted from transduced cells
- To compare uptake of *GBA* by untransduced cells using wild type and modified HIV-1 TAT protein transduction domains

4.2 Introduction

Although the primary cells to show a defect in GD are of the haematopoietic lineage, *GBA* is in fact a housekeeping gene and is expressed by almost all cell types at varying levels (378-380). It could therefore be beneficial to patients if enzyme expressed from the introduced gene could be used by non-haematopoietic cells within a tissue. This is of particular relevance in the bone microenvironment which contains osteoclasts, of haematopoietic lineage as well as osteoblasts and osteocytes which are mesenchymal in origin (84). Targeting bone could be especially beneficial in treating GD as the skeletal aspects of the disease remain a significant burden, even to patients taking ERT (152, 153, 156). However although it is known that the mannose receptor, used for uptake of GC by macrophages, is expressed during osteoclast development (387); it is not clear to what extent the receptor is capable of mediating protein uptake *in situ* so it may not be possible to obtain this cross-correction through the mannose receptor.

GC is an intracellular protein which is trafficked to the lysosome compartment by the receptor protein LIMP2 (23). Although it is not normally secreted, the *GBA* gene carries

an endogenous secretion signal and the protein appears to be secreted by cells lacking LIMP2. It was therefore hypothesised that if the protein is expressed at supranormal levels due to viral vector transduction, the secretion signal could be used to prevent the accumulation of excess enzyme within cells. If this is the case then the secreted enzyme could serve as a natural reservoir of functional protein for other cells throughout the body.

In correspondence with this hypothesis, it has previously been shown that GC is secreted from transduced cells and uptake of secreted protein can be mediated by endocytosis (388). However, this evidence is limited and the degree of uptake has been low.

The enzyme preparation used for ERT has been engineered to have side chains which are mannose-terminated so that it will be taken up by the macrophage mannose receptor (MMR) and thereby directly target the macrophage. In order to achieve this targeting, the enzyme produced by Genzyme must be post-translationally modified to ensure that the terminal residues are recognised by the MMR (109). Other enzyme preparations have a high proportion of mannose terminated side chains due to producer cell effects (taliglucerase alfa) (133) or culture medium additives (velaglucerase alfa) (129, 130). However, mannose-termination is only of use when the macrophage is the target cell; when the aim is to get the enzyme into a wide range of cells a less specific strategy is required.

One method used to achieve widespread delivery of therapeutic proteins is to fuse the protein to a generic protein transduction domain (PTD) which is capable of transducing protein across the plasma membrane by receptor-independent, and therefore non-specific, means. Fusion of *GBA* to a PTD could produce an enzyme variant which, if secreted, is able to enter other cells which may not have been directly corrected by the gene therapy.

One of the best characterised PTDs is that of the HIV-1 transactivator of transcription (TAT) protein transduction domain. The PTD from the TAT protein has been shown to work by stimulating protein uptake by lipid raft-mediated endocytosis (346, 347) which is ideal for lysosomal proteins, such as GC as the protein is ultimately delivered to the lysosome (4, 13, 14).

A previous study demonstrated that in an ERT setting, addition of the TAT PTD to the C-terminus of GC results in a significant increase in the amount of protein taken up by fibroblasts in culture compared to imiglucerase and wild type GC but that fusion to the N-terminus of the protein abolished protein expression (315). A subsequent study demonstrated the presence of two furin cleavage sites within the PTD which presents a possible explanation for the lack of expression from the N-terminal fusion (316). Furin is a ubiquitous endoprotease which recognises the sequence R-X(K/R)-R and is responsible for the release of mature proteins from their precursor form.

The authors who identified the furin cleavage sites within the TAT PTD produced a modified version which lacked the furin recognition sites (mTAT). This version of the PTD was used in this study in addition to the WT version (WT TAT) to compare the effect of N- and C-terminal fusion. The sequences of the wild type and modified domains are shown in Figure 4.5A. The following chapter will document the work using the GC-PTD fusion products.

4.3 Results

4.3.1 Developing assay for secreted protein

Neither of the GC assays used so far in this project are performed on supernatant but it was thought that the assay using the FDGlu substrate would be suitable for adaptation for use with supernatant samples using a plate reader within the group which has suitable filters.

There are two companies which produce the FDGlu substrate – Life Technologies (LT) and Marker Gene Technologies (MGT) – and substrate was purchased from both of them for testing. To begin with, levels of autofluorescence were measured when substrate was diluted in DMEM with or without foetal calf serum (FCS). FCS is added to culture medium because it contains a high concentration of growth factors and other components which aid in the propagation of cell lines. The substrate was tested in medium with and without FCS to ensure that there is no residual GC enzyme or other factor in the serum which may affect background fluorescence levels. Figure 4.1 shows that the substrate produced by MGT gave significantly higher levels of autofluorescence when added to medium and so it was decided to continue tests using the substrate from LT.

To establish whether the high levels of autofluorescence seen with the LT substrate could be reduced, the assay was repeated using a range of media types, including PBS and water to resuspend the substrate. A GC inhibitor (conduritol- β -epoxide; CBE) was added to the media samples to control for any intrinsic enzyme activity within the media. These experiments revealed that the substrate produces a similar amount of autofluorescence in all media types with or without CBE (Figure 4.2).

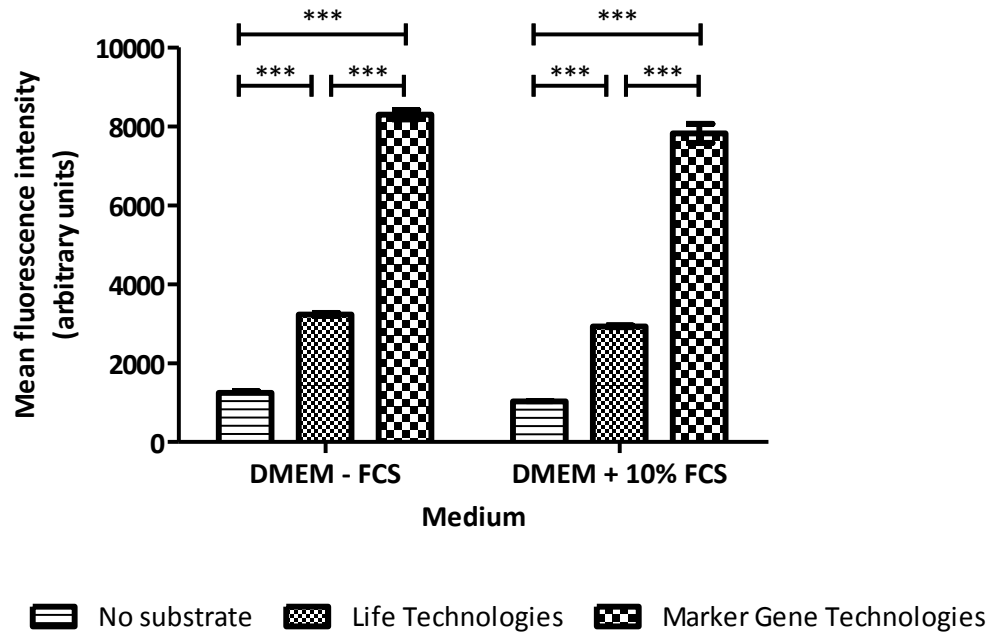


Figure 4.1 Comparison of the levels of autofluorescence produced by different preparations of FDGlu substrate in medium

To determine which of the two commercially available preparations of FDGlu substrate was most suitable for use in testing for GC activity in transduced cell supernatant, substrate produced by either Life Technologies or Marker Gene Technologies was incubated for 45 minutes in DMEM with or without foetal calf serum and then assayed for fluorescence levels. No enzyme was added so any detected fluorescence is a result of substrate autofluorescence. The substrate from Marker Gene Technologies produced significantly higher levels of autofluorescence and so it was decided to proceed using the substrate produced by Life Technologies. N=3, error bars show standard deviation. Significance was tested using one-way ANOVA with Bonferroni post-hoc test: ***= $p < 0.0001$.

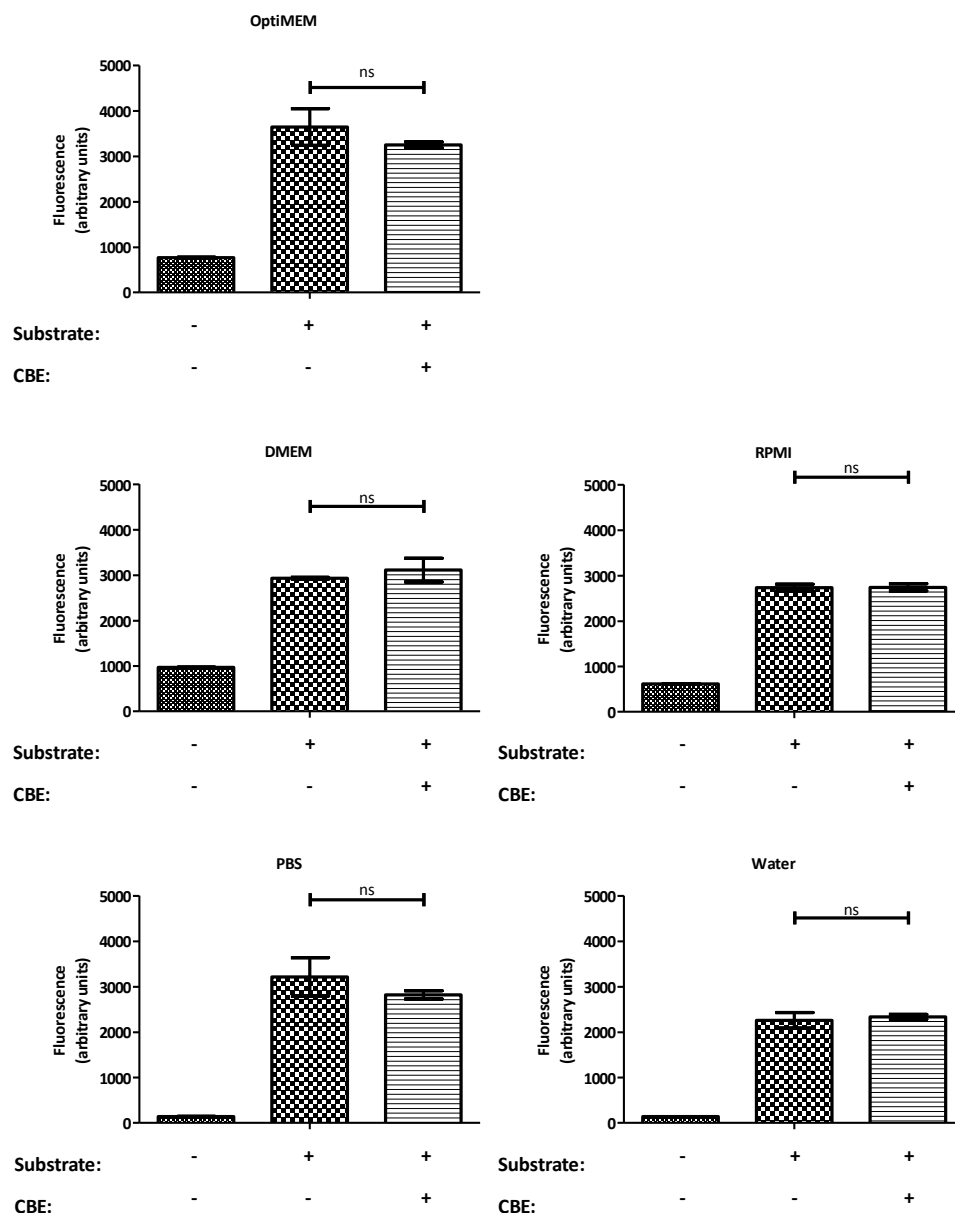


Figure 4.2 Autofluorescence of fluorescein- β -D-glucopyranoside in media with and without conduritol- β -epoxide

FDGlu substrate (LT) was incubated in a range of media types with or without the GC inhibitor CBE and fluorescence measured to determine the baseline signal from medium alone. No enzyme was added to the reactions so the detected fluorescence is caused by autofluorescence of the substrate as confirmed because levels of fluorescence do not decrease when the enzymatic inhibitor CBE is added. N=3, significance tested by one-way ANOVA with Dunnett's post-hoc test, error bars show standard deviation ns = $p > 0.05$.

Despite high levels of FDGlu substrate autofluorescence, when the ERT preparation velaglucerase alfa (Shire HGT) was diluted in water it was possible to detect enzyme levels of 10^{-1} – 10^{-2} units/ml which is equivalent to protein concentrations of 2.5×10^{-3} – 2.5×10^{-4} mg/ml (Figure 4.3A). Comparatively, when the same enzyme dilutions were used in the 4MUG assay, detection limits extended from 10^{-2} – 10^{-5} units/ml or 2.5×10^{-4} – 2.5×10^{-6} mg/ml which is a greater range and of more use in this project where it is likely that protein concentrations are going to be lower. For this reason it was decided that secreted protein levels would be assayed using the 4MUG assay only.

Western blotting of the diluted velaglucerase alfa samples was much less sensitive than either assay and could barely detect enzyme at levels below 0.625 units/ml (1.6×10^{-2} mg/ml) (Figure 4.3B).

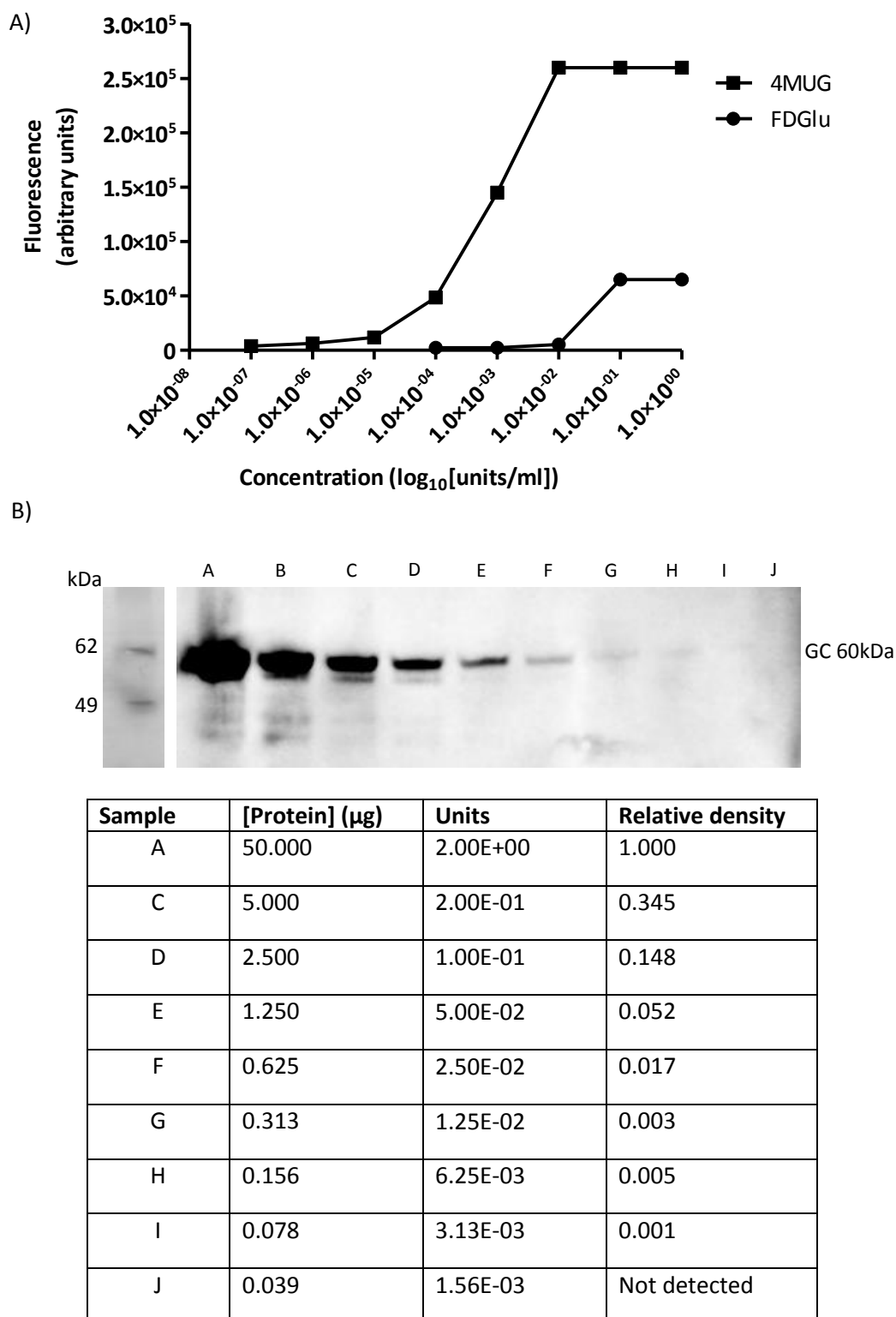


Figure 4.3 Detection of velaglucerase alfa by enzymatic assay and western blot

A) Velaglucerase alfa (VPRIV) was diluted in water and used to test the two fluorometric assays described previously. The 4MUG assay was found to be the more sensitive of the

two as it was able to detect dilutions of 10^{-5} units/ml whereas the FDGlu assay could only detect enzyme at a 100 times greater concentration. B) The sensitivity of the GC antibody used in this project was tested using dilutions of velaglucerase alfa and quantified using ImageJ software (table).

4.3.2 Cloning vectors

4.3.2.1 pHR-SFFV-GBA-mTAT and pHR-SFFV-mTAT-GBA

Fusion of the HIV-1 TAT protein transduction domain (PTD) to *GBA* was achieved using a polymerase chain reaction (PCR) strategy (Figure 4.4). Primers were designed which contained part of the PTD and part of the *GBA* sequence. Overlapping PCR was performed using Pfu DNA polymerase to generate a full length *GBA* sequence with an in-frame fusion of the modified TAT PTD on either the N- or C-terminus (Figure 4.5). The N-terminal PTD sequence was placed after the endogenous GC secretion signal which is cleaved from the protein.

The full-length fragment was purified by gel electrophoresis. The fragment was then subcloned into the TOPO-blunt vector to provide an easily amplified source of DNA for further cloning. The primers used contain restriction enzyme sites at either end of the gene so that it could be easily excised from the TOPO vector and cloned into the pHR lentiviral backbone. To perform this step the *GBA* fusion fragment was excised using the restriction enzymes EcoRV and XhoI. The plasmid backbone was digested with BamHI and blunted using DNA polymerase I Large Fragment (Klenow) to give a blunt end which is compatible with DNA digested by EcoRV. The backbone band was purified from the agarose gel following electrophoresis and subsequently digested with XhoI. The two fragments were then ligated using T4 DNA ligase and transformed into bacteria. The vectors produced are shown in Figure 4.5B.

4.3.3 pHR-SFFV-GBA-WT TAT

A similar strategy to the one detailed above was employed to produce a construct carrying *GBA* fused to the wild type TAT PTD at the C-terminus (Figure 4.5B). An N-

terminal fusion of the WT TAT PTD to *GBA* was not produced because of the earlier work which showed that this fusion protein would not be expressed (315).

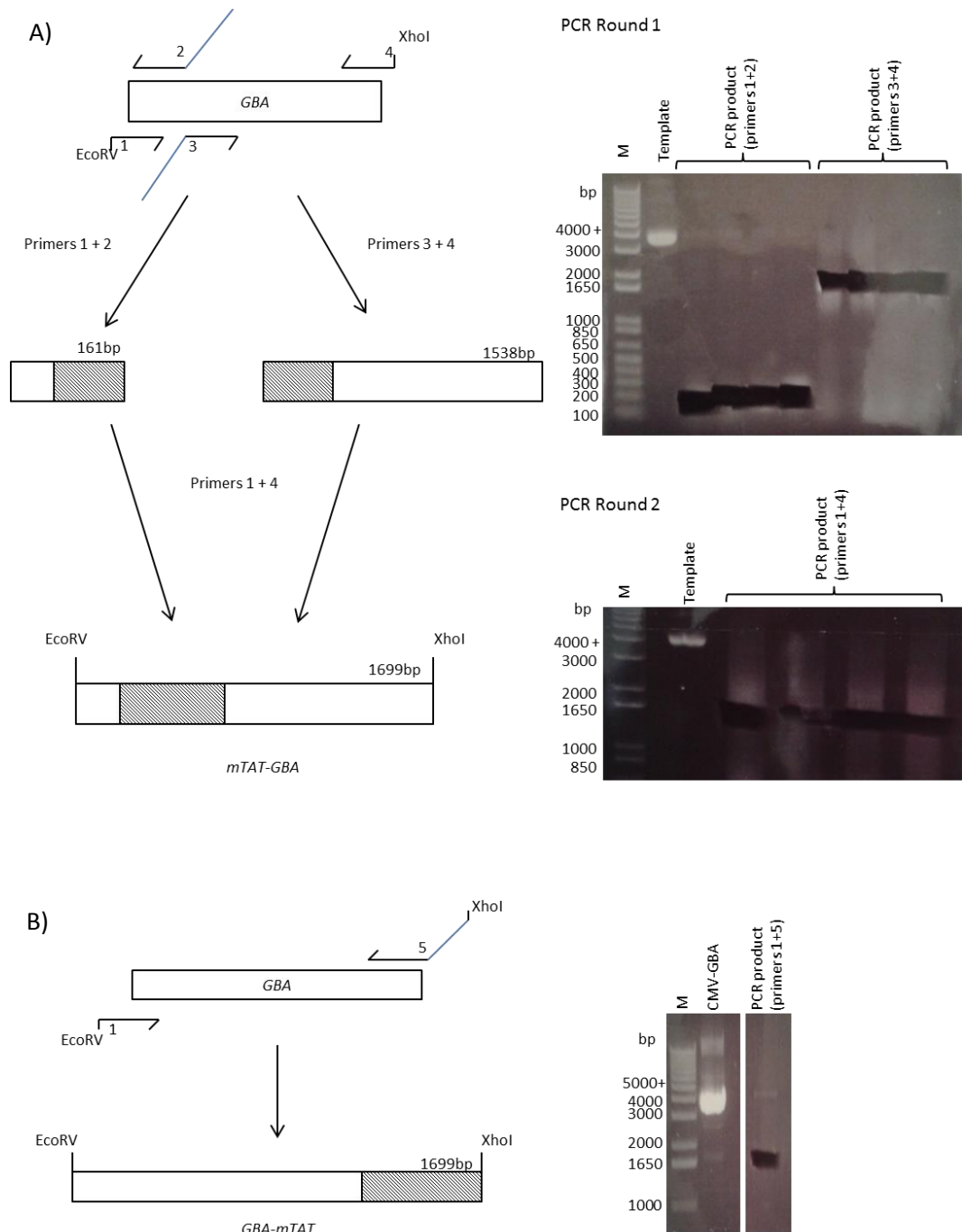


Figure 4.4 Cloning strategies to generate fusion constructs

A) N-terminal fusion of PTD to *GBA*. Two rounds of PCR are performed, the first using primers 1+2 or 3+4 and with the WT *GBA* cDNA as template produced two products, one which is the *GBA* leader sequence fused to a C-terminal PTD sequence and the second being the rest of the *GBA* gene with the complementary PTD sequence on the N-terminus. Both products are purified and used as template in the second round of PCR which uses primers to the N- and C-terminus of *GBA*. The complementary PTD sequences

present on the round one products anneal during synthesis producing a complete template and allowing read through from primer 1 to 4. The resulting product of 1699bp in length is purified and cloned into pHR using EcoRV and XhoI restriction enzymes. B) Shows fusion of the TAT PTD sequence to the C-terminus of the *GBA* cDNA by a single round of PCR using a primer containing the full length PTD (primer 5). In both figures the blue line indicates the TAT sequence within the primer and the shaded bar corresponds to the TAT sequence within the PCR product.

A)	Name	Amino acid sequence
	Wild type TAT (WT TAT)	YGRKKRRQRRR
	Modified TAT (mTAT)	YARKAARQARA

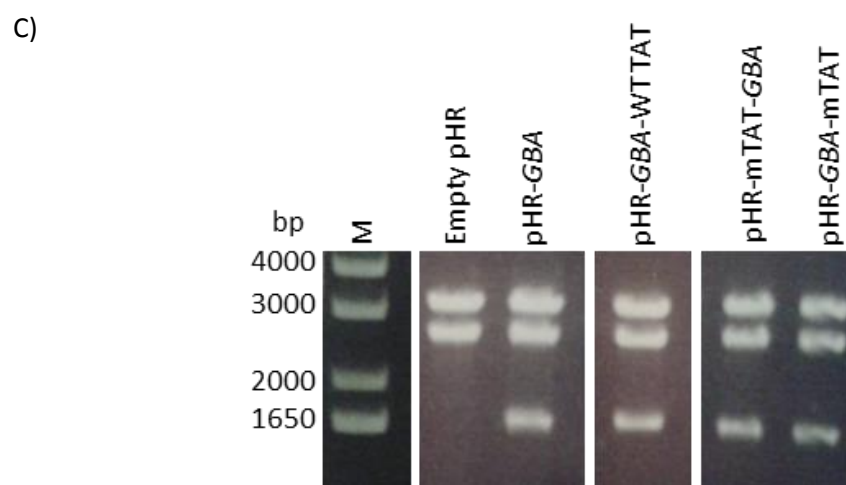
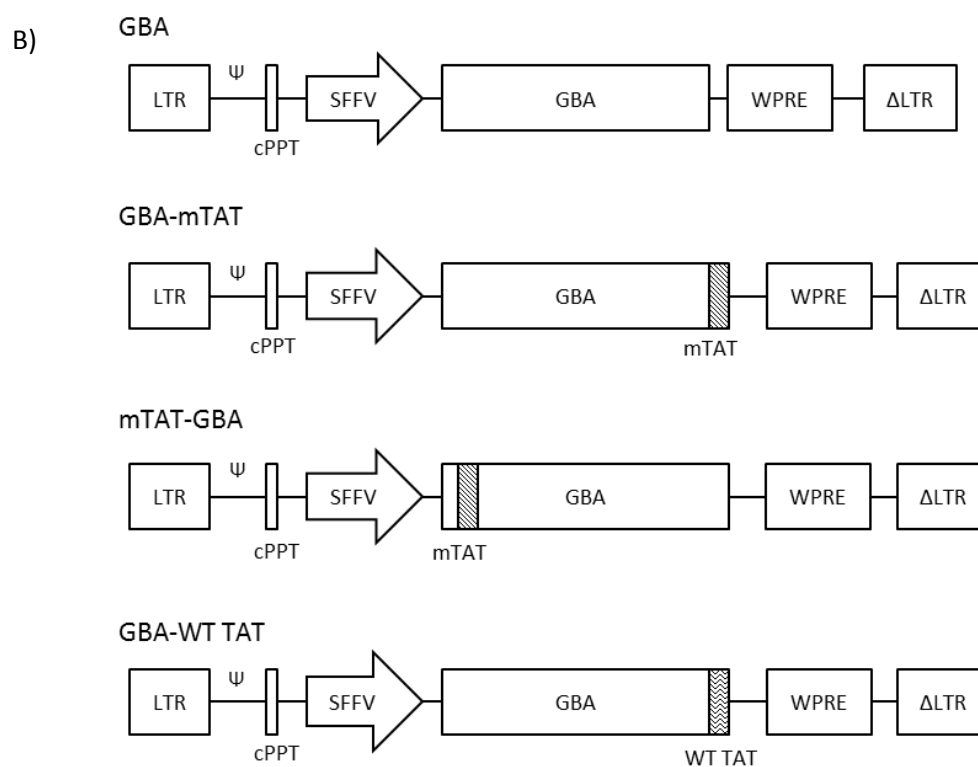


Figure 4.5 Schematic showing vectors produced

A) The sequences of the two versions of the TAT protein transduction domain used in this work. mTAT has been modified to remove two furin cleavage sites (underlined). B) Schematic of the produced pHR-*GBA* constructs. LTR: long terminal repeat; Ψ : packaging signal; cPPT: central polypurine tract; SFFV: spleen focus-forming virus promoter; WPRE: woodchuck hepatitis post-transcriptional regulatory element; Δ LTR: self-inactivating LTR. C) Electrophoresis gel showing empty pHR backbone (1), pHR-*GBA* (2), pHR-mTAT-*GBA* (3), pHR-*GBA*-mTAT (4) and pHR-*GBA*-WT TAT (5) digested with the restriction enzyme HindIII. The lowest band in lanes 2-5 corresponds to 1.6kB which confirms the addition of the *GBA* gene. The electrophoresis gel used here was a 1% agarose gel meaning that the addition of the TAT domains is not visible and therefore had to be confirmed by sequencing. However had a higher percentage agarose gel been used, the addition of the PTD would have been detectable by electrophoresis. Sequencing would still have been required to ensure that the correct sequence had been added.

4.3.4 The addition of the protein transduction domain does not affect expression or activity of GC

Vectors were tested in GD-derived fibroblasts to ensure that the addition of the PTD does not have an adverse effect on protein expression or activity. Fibroblasts were transduced at an MOI of 50 and cultured for one month to provide enough material to perform western blots and GC assay in parallel. The results showed that cells transduced with the PTD containing vectors yielded a protein of the same molecular weight as the WT construct (Figure 4.6A). Despite the addition of a transduction domain, an increase in protein size was not expected. This is because the PTD contains only 11 amino acids and an increase of this size is unlikely to be detectable by western blot.

The activity of the protein with the added PTD was confirmed when transduced fibroblasts were harvested for use in the 4MUG assay. Transduction with all three of the vectors restored enzyme activity in the fibroblasts to within the range designated as healthy (Figure 4.6B). Transduction with a control vector expressing eGFP had no effect on enzyme activity levels confirming that the effect is vector specific.

The western blot in Figure 4.6A seems to suggest that there is less GC protein being produced from the mTAT-GBA construct compared to the other two GC plasmids. However this is most likely due to a loading error as the amount of GAPDH, an internal control protein is also reduced compared to the other samples on the blot and this is supported by the data shown in Figure 4.6B which shows that cells transduced with all three GBA carrying vectors have equal levels of enzyme activity post-transduction.

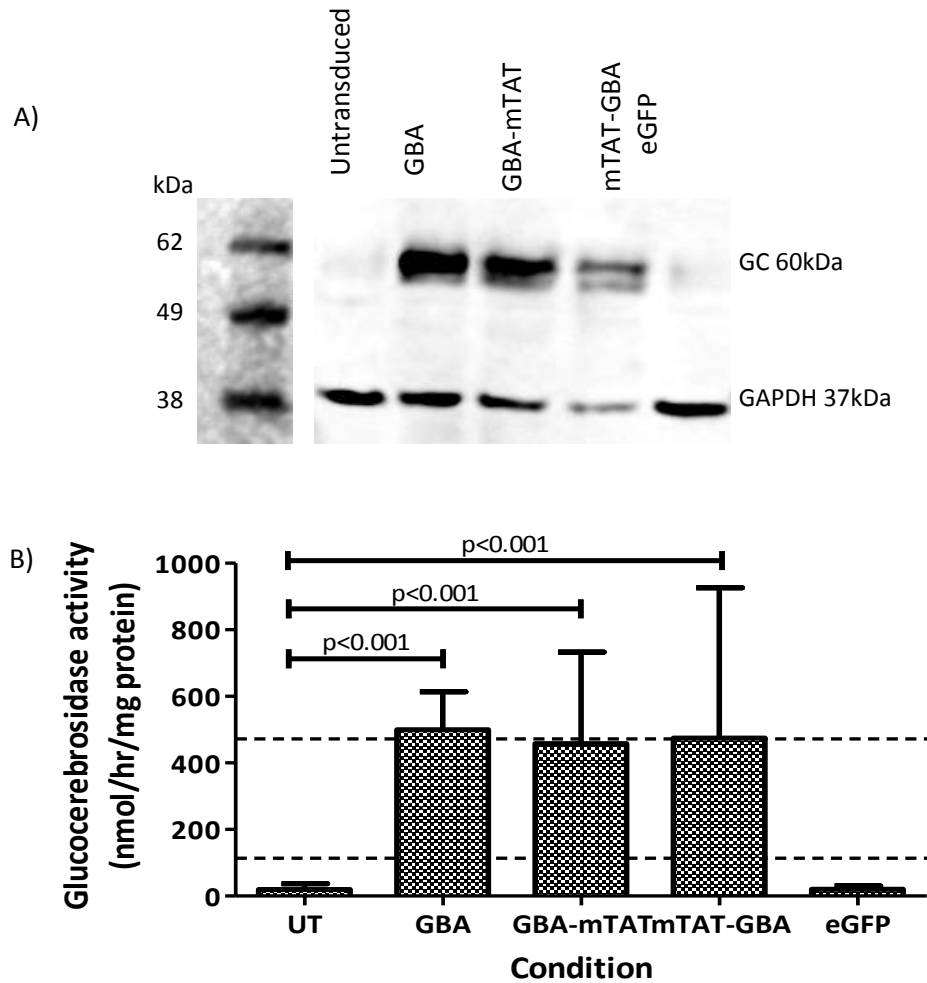


Figure 4.6 Addition of the mTAT PTD does not affect GC expression or activity

GD-derived fibroblasts were transduced with vectors carrying either *GBA* or *eGFP* and harvested one month post-transduction to assess GC expression and activity. A) Western blot of transduced cells shows production of a protein of similar molecular weight to WT GC from all constructs. B) 4MUG assay shows equal activity levels in WT and PTD fused forms of GC. Error bars show standard deviation, $n=7$ Significance was tested using one-way ANOVA with Dunnett's post-hoc test. The dashed lines indicates the range designated as healthy levels of GC activity.

4.3.5 Transduced cells secrete GC protein via an endogenous secretion signal

It is known that the GC protein contains an endogenous secretion signal however it is not commonly thought of as a secreted protein. It is likely that this is because GC is expressed at a relatively low level in all tissues and is therefore retained within the cell (378, 389). It was therefore hypothesised that cells which produce a very high level of GC as a result of transduction by *GBA* carrying lentivirus would secrete excess protein into the extracellular environment.

After validation of the antibody used for western blots an experiment was performed to determine whether GC is secreted by transduced cells. HEK293T cells transduced at an MOI of 100 were seeded in a dish and after 72 hours the cell supernatant was harvested and western blots and 4MUG assays were performed (Figure 4.7). An MOI of 100 was used despite evidence above showing that an MOI of 50 is sufficient to produce healthy levels of GC activity because it was thought that GC would only be secreted if it is expressed at supraphysiological levels. Figure 4.7 clearly shows that the GC protein is being secreted by transduced cells at high level with the highest level of secretion from cells transduced with the *GBA*-mTAT vector although the reason for this is unclear especially as intracellular protein levels are the same with all three constructs (Figure 4.6). An expansion of this work would be to test lower levels of transduction and see at what point secretion can be observed.

A time course run over 14 days on cells transduced with the WT *GBA* construct showed that enzymatic activity can be detected in supernatant from seven days post-transduction and continued to rise throughout the period of the study (Figure 4.8). Although *GBA* expression can be detected within the cell from day one post-transduction, it is likely that secretion cannot be detected from as early because the amount of protein within the cell

has to accumulate to a point where secretion is required to reduce intracellular levels. This may also help to explain the decrease in intracellular protein levels post-day three as excess protein is removed.

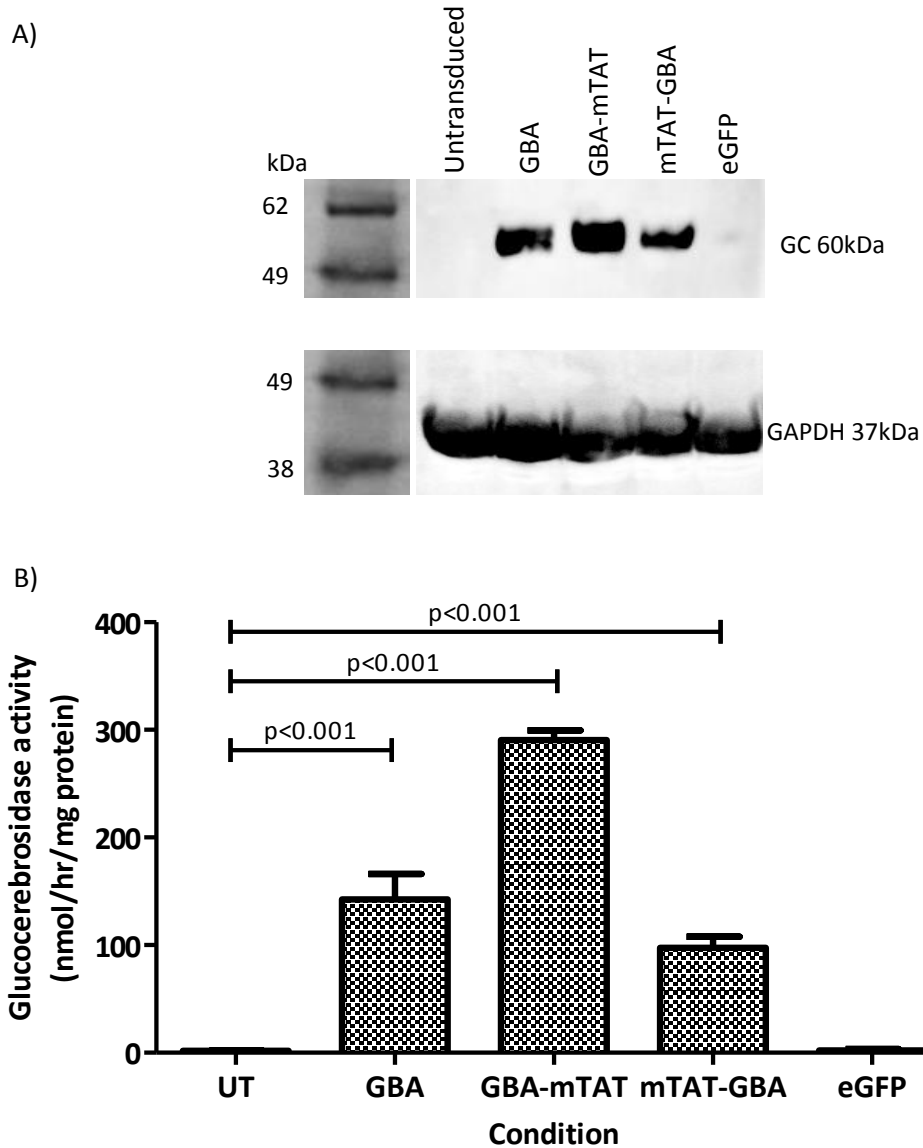


Figure 4.7 Secretion of GC from transduced cells

The supernatant of HEK293T cells 72 hours post-transduction was harvested and tested for the presence and activity of GC. A) Western blot showing GC can be detected in the supernatant of cells transduced with *GBA* vectors at high MOI. The lower panel shows BSA as a loading control. B) The 4MUG assay run on the same samples as in A, showing a significant increase in the enzymatic activity of supernatant from transduced cells compared to untransduced cell supernatant. Error bars show standard deviation, $n=3$. Significance tested with one-way ANOVA with Dunnett's post-hoc test.

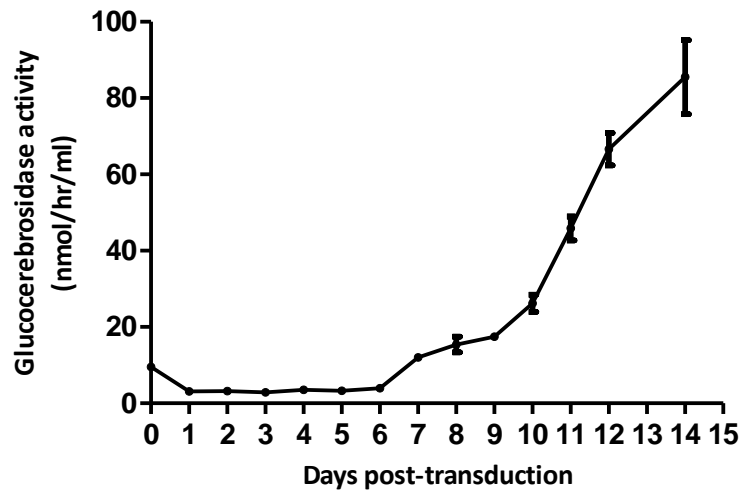


Figure 4.8 A 14-day time course showing GC activity within the supernatant of transduced cells reveals secretion of enzyme

HEK293T cells were transduced with *GBA* virus at an MOI of 100 and cultured for 14 days with samples of supernatant harvested every day. A 4MUG assay run on the harvested samples showed that secreted protein can be detected from around day 6 post-transduction and continues to accumulate over the 14 days (n=3). Error bars show standard deviation.

4.3.6 Uptake of GC can be mediated by protein transduction

domains

Figure 4.9A shows the experimental protocol followed to assess cross-correction of untransduced cells by secreted GC enzyme. HEK293T were transduced at MOI 100 and allowed to grow for 14 days so that they would be a point when enzyme secretion was at a high level. 10^5 cells were then seeded in a 12-well plate and left to adhere overnight. The next day a transwell insert with pore size $0.4\mu\text{m}$ was added to the top of the well which would allow the exchange of medium and low molecular weight proteins only between the two areas of the well. GD patient-derived LCLs were added to this insert after 2-4 hours and cultured without medium change for 72 hours. After this time period, cells were harvested, washed and assayed for enzyme uptake by western blot and 4MUG assay.

Western blot was unable to detect GC enzyme, presumably because it was present only in small quantities but the assay detected a significant rise in enzyme activity within LCLs cultured with cells expressing the *GBA*-mTAT and *GBA*-WT TAT constructs (Figure 4.9B).

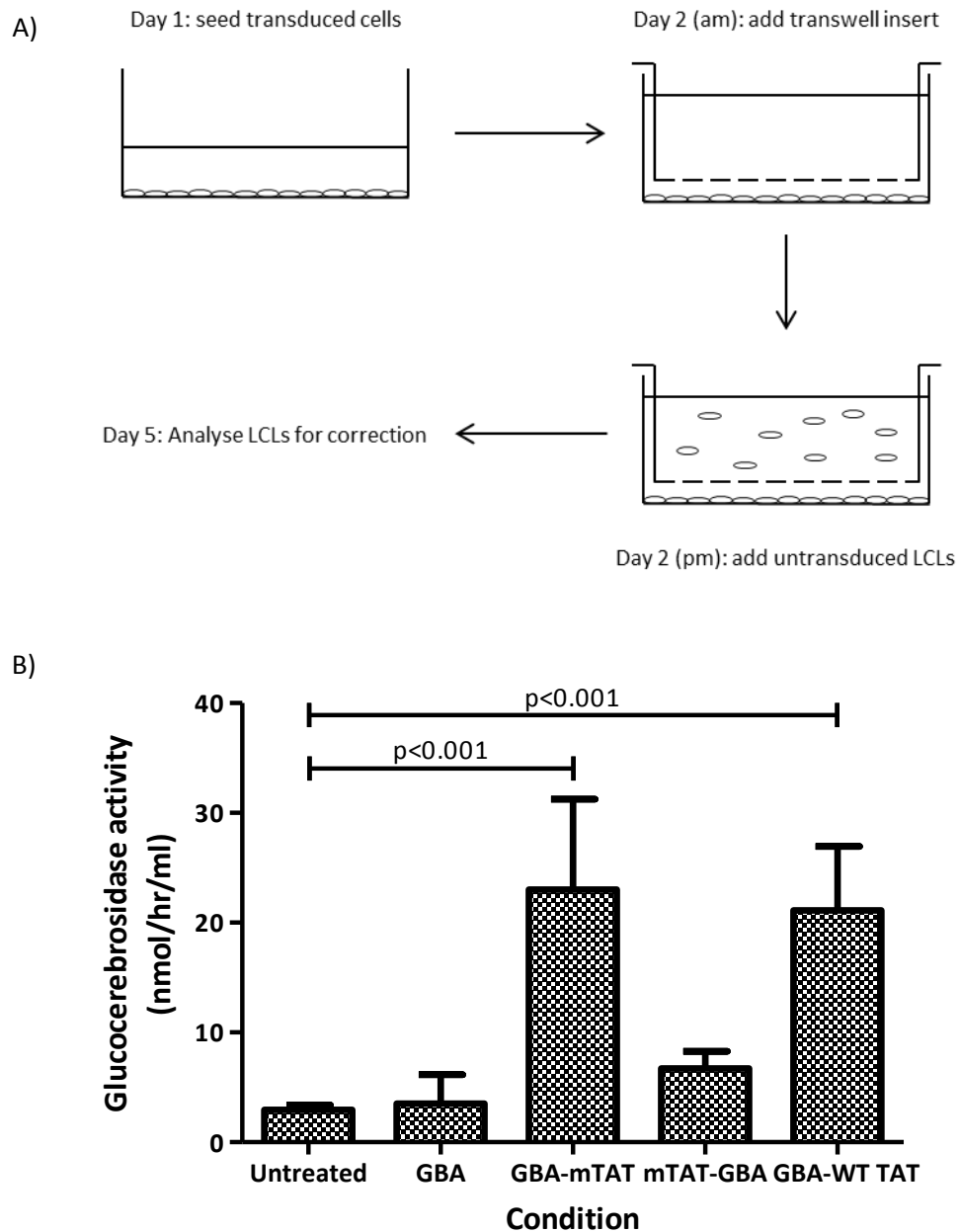


Figure 4.9 Cross-correction of $GBA^{-/-}$ LCLs by protein secreted from transduced HEK293T cells

A) Schematic showing the methodology involved in cross-correction experiments. B) Co-culture with HEK293T cells transduced with *GBA*-mTAT and *GBA*-WT TAT significantly increases the glucocerebrosidase activity in GD-derived LCLs. Other constructs do not have the same effect. Analysed with one-way ANOVA and Dunnett's post-hoc test (n=3).

4.4 Summary

In this chapter the work described in chapter 3 has been further developed by modifying the *GBA* cDNA to include a protein transduction domain derived from the HIV-1 TAT protein. This was done to investigate the feasibility of using transduced cells as a reservoir of protein which, when secreted, could be taken up by neighbouring, untransduced cells to correct the metabolic defect throughout the body. This was investigated because *GBA* is a housekeeping gene and is expressed ubiquitously, albeit at low levels, and it could therefore be beneficial to correct multiple cell types. This is of particular relevance in the bone microenvironment. As will be described in the next chapter it is thought that the osteoclast, a cell of haematopoietic origin, is a major contributor to skeletal disease in GD but it is also possible that other cells such as the osteoblast and plasma cell may also be implicated in the development of skeletal complications (80, 89). As the osteoclast is haematopoietic in origin, it would be directly corrected by gene therapy; however, a reservoir of secreted GC could be of benefit to the other cells in the environment and thereby help alleviate symptoms.

It has been shown here that fusion of the protein transduction domain is only successful in mediating uptake of GC when it is added to the C-terminus of the enzyme. Fusion of the PTD to the N-terminus does not mediate an increase in the enzymatic activity of co-cultured cells. It is possible that this could be due to folding of the protein resulting in the PTD being internal to the protein and therefore not interacting with the untransduced cell type. However this requires further investigation before any conclusions can be drawn.

5 Investigating gene therapy as a potential treatment for the osteoclast

5.1 Aims

- To show that gene modified haematopoietic progenitor cells can generate osteoclasts expressing an introduced gene.
- To investigate the phenotype of osteoclasts derived from bone marrow cells from the GD type II mouse model.

5.2 Introduction

One of the most significant aspects of type I Gaucher disease (GD) in the post-ERT era is bone disease; 75-90% of patients still experience skeletal symptoms to some extent and in some people the condition can be severely debilitating (50, 77, 390). Although it has been shown that bone disease can respond to ERT, it can take several years to show any improvement (152, 153, 157) which is likely to be because the therapy is directed to target the macrophage rather than to treat the bone directly (109). A survey conducted as part of this project, and with the assistance of the Gauchers Association, supported this finding as 60% patients cited bone pain/skeletal symptoms as having a significant impact on their lives despite taking ERT (Figure 5.1).

Another challenge to treating the skeletal aspect of type I GD is that the mechanism of the disease remains unclear although recent data suggests that it may be due to increased differentiation and activity of the osteoclast (86). If this is the case then gene therapy could offer a method of preventing bone disease and potentially directly correcting it. The osteoclast is a cell of hematopoietic origin (Figure 1.7) and so correction of HSCs by gene therapy would lead to the differentiation of GC positive osteoclasts *in*

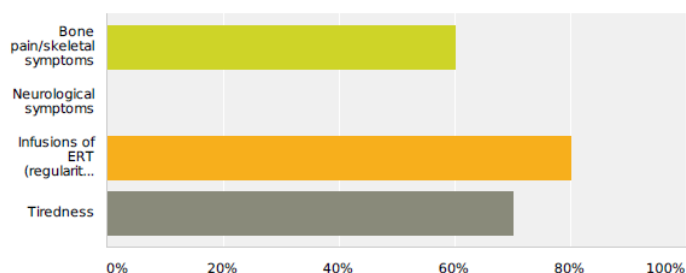
vivo. However very little work has been conducted to test the effect of gene therapy on osteoclasts and the differentiation of gene corrected HSCs to bone cells.

One of the reasons that the molecular basis of skeletal pathology remains unclear is that over the years there has been a shortage of animal models which successfully recapitulate the symptoms of GD and none which mimic the skeletal problems seen in patients. A paper published in 2010 described a new mouse model which does show significant osteonecrosis and osteopenia; two of the major pathologies found in GD. These findings were attributed to defective osteoblastogenesis while osteoclast differentiation appeared normal (89). It is therefore possible that this mouse model will not serve as an accurate representation of the cellular defect in human patients as there is emerging evidence for osteoclast involvement in the human disease (86).

Recently a mouse model has been developed by Stefan Karlsson at Lund University, Sweden which is a conditional knock-out of the *GBA* gene and displays severe neuronopathic GD (184). Although this mouse is not a perfect model for the work performed in this project, the strain was kindly made available to Simon Waddington (UCL Institute for Women's Health) who has established a colony. Affected mice must be culled at the age of fourteen days as they develop continuous seizures and paralysis so they do not develop bone disease but, as cells do not produce GC, they could be a useful model for studying pathology *in vitro*.

Q8 We would like to know what, in your opinion and experience, are the most difficult aspects of Gaucher disease to live with:

Answered: 10 Skipped: 0



Answer Choices	Responses
Bone pain/skeletal symptoms	60% 6
Neurological symptoms	0% 0
Infusions of ERT (regularity/procedure)	80% 8
Tiredness	70% 7
Total Respondents: 10	

Figure 5.1 Surveyed type I patients find skeletal problems a significant aspect of living with Gaucher disease

In a survey of a small group of type I GD patients, 60% reported bone pain and other skeletal symptoms as being the most difficult aspect of GD to cope with. Other reported difficulties include the frequency with which ERT must be administered and tiredness which is most likely associated with residual anaemia. No respondents reported neurological symptoms but this was expected as type I disease is classically ‘non-neurological’.

5.3 Results

5.3.1 Comparison of osteoclast number in BL6 and CD1 mice

Laboratory mice are inbred strains which have been engineered to have certain characteristics. While many of these characteristics are well documented, the ability to produce osteoclasts is not usually recorded. Before any investigative work could be performed it was necessary to assess the rates of osteoclastogenesis in the two mouse strains used in this study – BL6 and CD1. It is important to establish whether there is a difference in rate of osteoclast production for two reasons. Firstly, to know whether it would be feasible to compare work performed in different strains and second, because the disease may affect different strains to an unequal amount. The procedure used to differentiate osteoclasts from bone marrow is shown in Figure 5.2A, and the results from the two strains in Figure 5.2B.

The results showed that the CD1 strain, which was used to breed the *GBA*^{-/-} mouse, is capable of producing more osteoclasts *in vitro* although this result is not entirely conclusive as only one mouse was used in each condition. However a high rate of osteoclastogenesis is beneficial to this study as it indicates that the CD1 background will be more sensitive to variation. This result indicates that it may be a suitable model for investigating differences in osteoclastogenesis between wild type and knock-out mice.

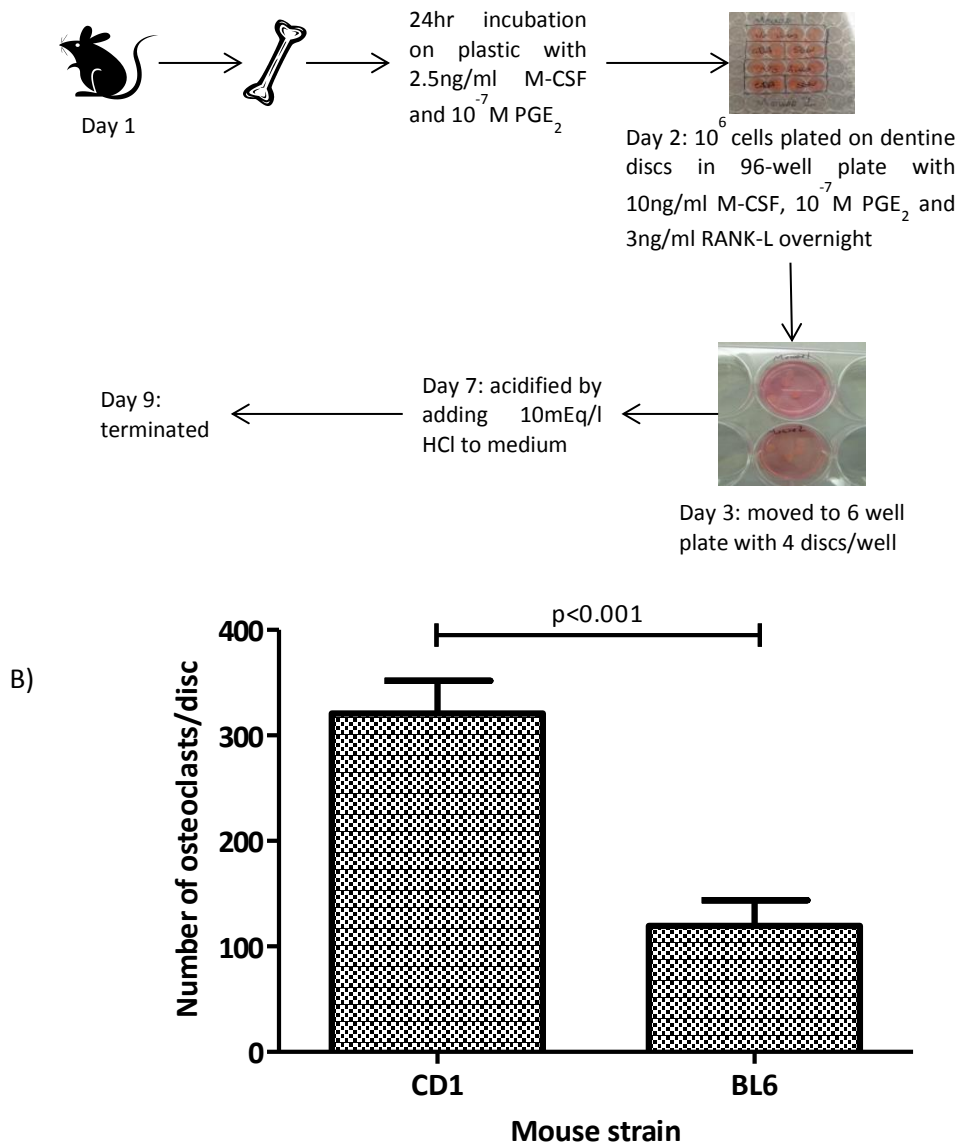


Figure 5.2 *in vitro* osteoclast differentiation differs in mice of different strains

A) Bone marrow was harvested post-mortem from mice of the BL6 or CD1 strain and used to generate osteoclasts *in vitro*. After nine days the cultures were terminated and stained for tartrate resistant acid phosphatase (TRAP) activity to identify osteoclasts. Osteoclasts were defined as large, TRAP positive, multinuclear cells. B) Osteoclast numbers generated from bone marrow of two different mouse strains. One mouse was used in each condition and the error bars show the standard deviation of number of osteoclasts on culture discs (n=12). An unpaired t-test generated a p value of <0.001 indicating a significant difference between strains.

5.3.2 Osteoclasts can be derived from transduced bone marrow

There is limited evidence available that modified bone marrow can differentiate into osteoclasts when transplanted into a recipient and so it was necessary to confirm that genetic modification of HSCs did not impair their ability to generate osteoclasts. It is important to note that a cell can only be deemed a true osteoclast when it has been differentiated on a physiological substrate, such as animal bone. For this reason cells which have been differentiated on glass and identified by morphology are referred to in the text as 'osteoclast-like cells'.

Bone marrow was harvested from the long bones of mice that had been transplanted with HSCs transduced by a lentivirus carrying the gene for green fluorescent protein (*eGFP*). Harvested bone marrow was differentiated into osteoclast-like cells *in vitro* (Figure 5.3B) on glass coverslips and stained for nuclei and actin. An osteoclast-like cell was defined as a large, multinuclear cell with a defined actin ring.

Differentiation of true osteoclasts from the harvested marrow was confirmed by performing the same protocol but culturing bone marrow on dentine discs to provide a substrate for resorption. Lysis of the cells after fixation revealed resorption pits made by active osteoclasts (Figure 5.3C) suggesting that subjecting bone marrow to viral transduction does not limit the capacity for deriving functional osteoclasts. However, it was not possible to measure fluorescence of cells cultured on dentine so it is not possible to conclude that the resorption pits were formed by osteoclasts differentiated from transduced cells, only that bone marrow which has been exposed to virus can retain its capacity to differentiate into osteoclasts.

The bones which had been stripped of their marrow were macerated and the resulting cell suspension was plated on glass coverslips and stained for actin and nuclei to identify osteoclasts which had differentiated *in vivo*. Using this method it was possible to identify

GFP positive osteoclasts from transplanted mice used which confirmed that transduced HSCs are able to differentiate into osteoclasts *in vivo* (Figure 5.4).

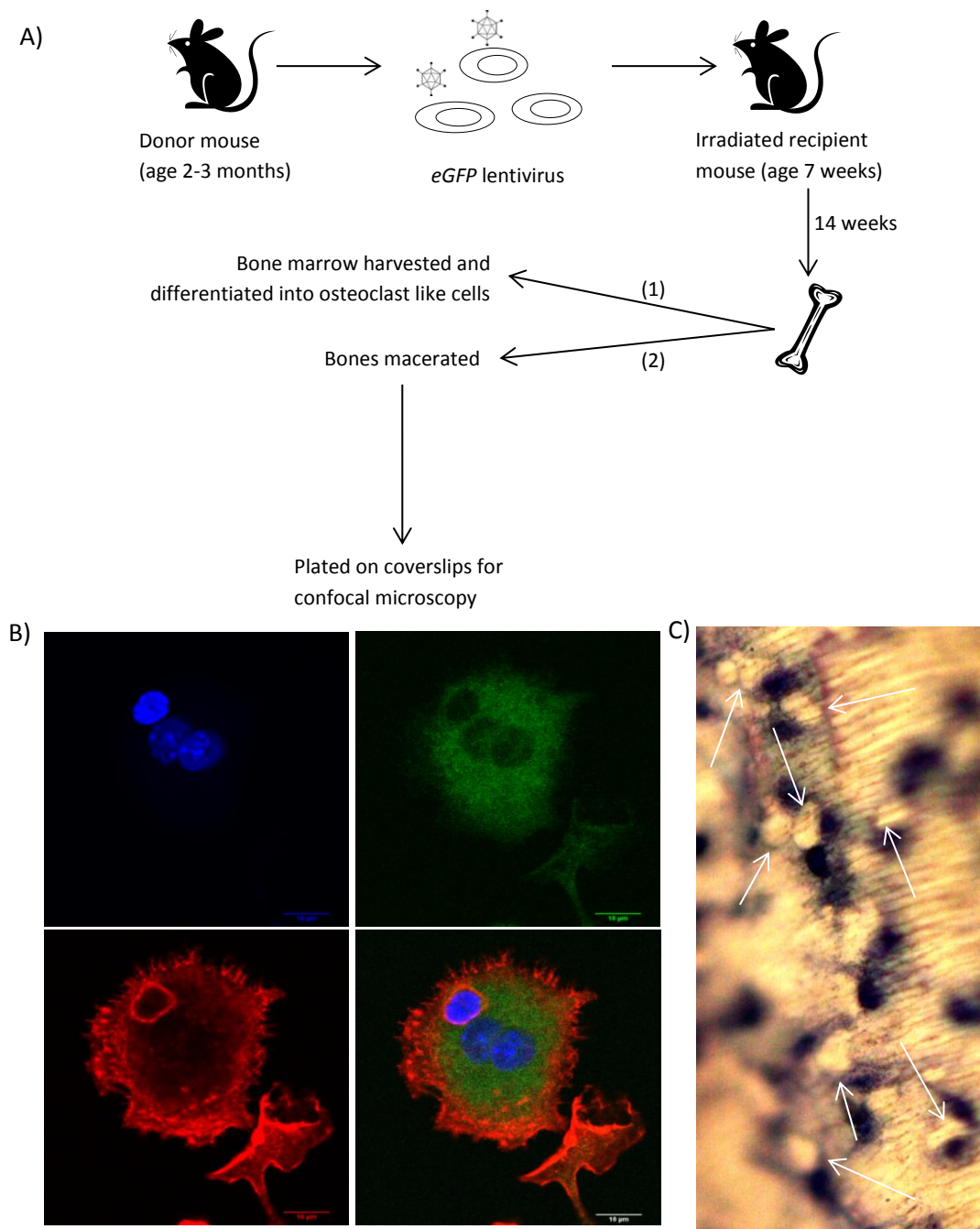


Figure 5.3 Osteoclasts can be derived from a genetically modified, autologous bone marrow transplant

A) Schematic showing the process of bone marrow modification, transplant and osteoclast experiments. This procedure up to and including culling the mice was performed by Marlene Carmo and Maria Alonso-Ferrero (UCL Institute of Child Health). After culling the long bones were extracted and used for two experiments: (1) Bone marrow was harvested and differentiated into osteoclast or osteoclast-like cells *in vitro*

and (2) the bones were macerated, plated on glass coverslips and stained to identify osteoclasts which had derived from transduced bone marrow *in vitro* (Figure 5.4). B) Confocal image of an osteoclast-like cell differentiated *in vitro* from harvested bone marrow (1) on glass coverslips. The actin image shows the characteristic actin ring around the edge of the cell and DAPI staining reveals it to be multinucleated. The cell can be identified as having derived from transduced bone marrow as it is expressing eGFP C) Light micrograph of dentine disc on which osteoclasts were cultured showing pits (arrows) caused by the bone resorption activity of functional osteoclasts (20x magnification).

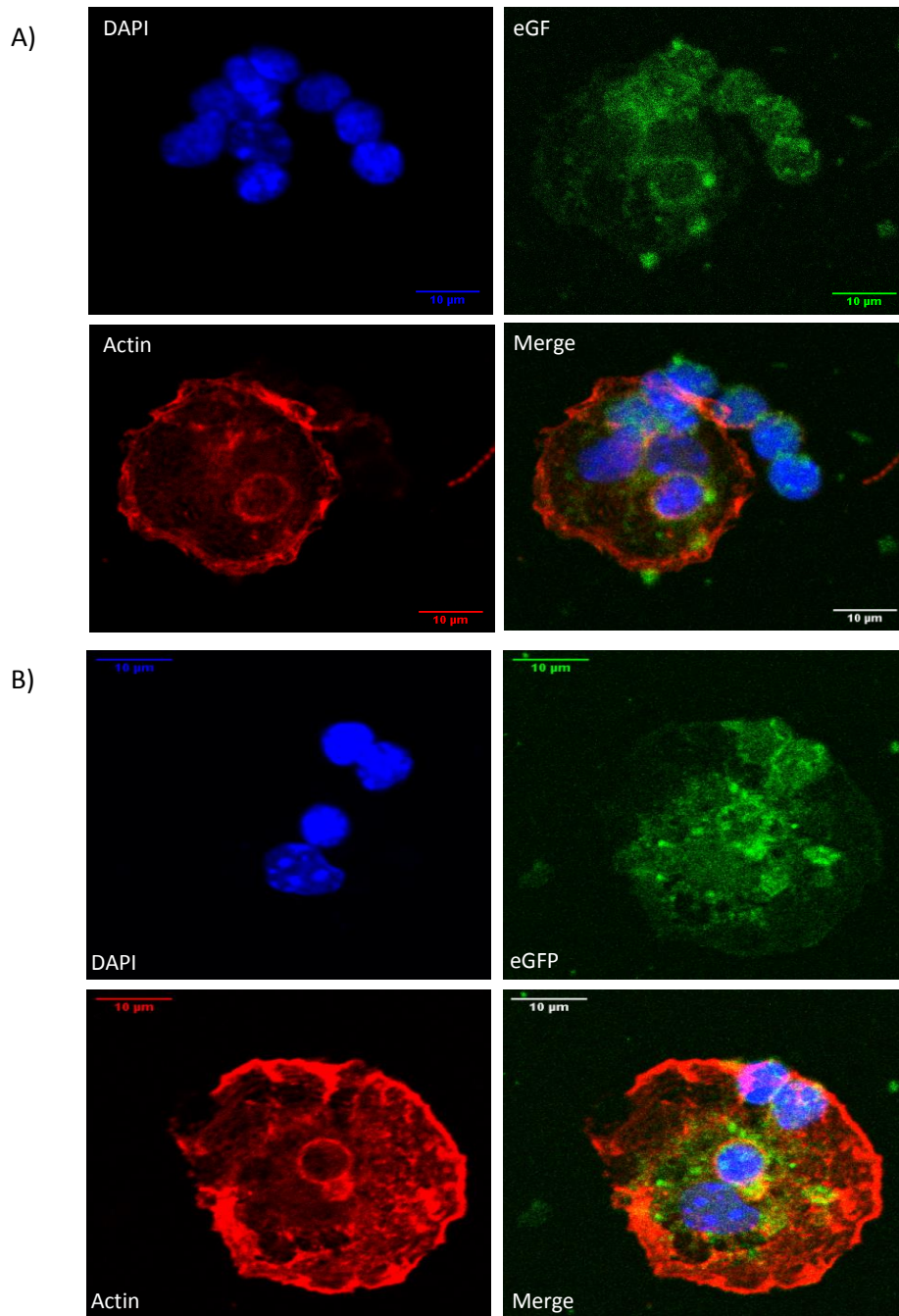


Figure 5.4 *in vivo* osteoclasts from mice transplanted with eGFP transduced bone marrow.

Bones of transplanted mice were removed, macerated (as shown in Figure 5.3A (1)) and the cell suspension plated on glass coverslips to identify osteoclasts which had differentiated *in vivo* from transduced HSCs. Shown here are images of two cells stained for nuclei and actin.

5.3.3 Osteoclast numbers in healthy vs GD mice

It is known that Gaucher disease patients develop bone problems later in life (50) and there is some evidence to suggest that this may be due to osteoclast hyperactivity (86) which may result in increased bone resorption accounting for the osteonecrosis and fractures common in patients.

Data published recently showed increased osteoclast differentiation and activity in peripheral blood mononuclear cells (PBMCs) from type I Gaucher patients *in vitro* compared to healthy control (86) but this has been contradicted by *in vitro* data using bone marrow (BM) from a type I mouse (89). The development of mouse models may be of use in resolving this issue and the current study took advantage of a type II model (184) to investigate the osteoclast phenotype.

BM from the knock-out mice was harvested when the mice were sacrificed at two weeks of age and was differentiated on glass coverslips to produce osteoclast-like cells *in vitro*. Differentiated cells were treated with stains for the nucleus and actin to identify osteoclasts (Figure 5.5A). True osteoclasts were also differentiated on dentine discs so that the activity of the cells could be assayed by comparing number of bone resorption pits produced by GD and control mice (Figure 5.5B).

The results showed that while there is no difference in the potential for knock-out bone marrow to differentiate into osteoclast-like cells, the $GBA^{-/-}$ osteoclasts appear to be much more active. This can be seen as there is no significant difference in the number of osteoclast-like cells produced on glass coverslips between the conditions but there is a significantly higher number of resorption pits seen on the discs from knock-out cultures indicating that the resorption capacity of the differentiated $GBA^{-/-}$ osteoclasts is significantly greater than their WT counterparts.

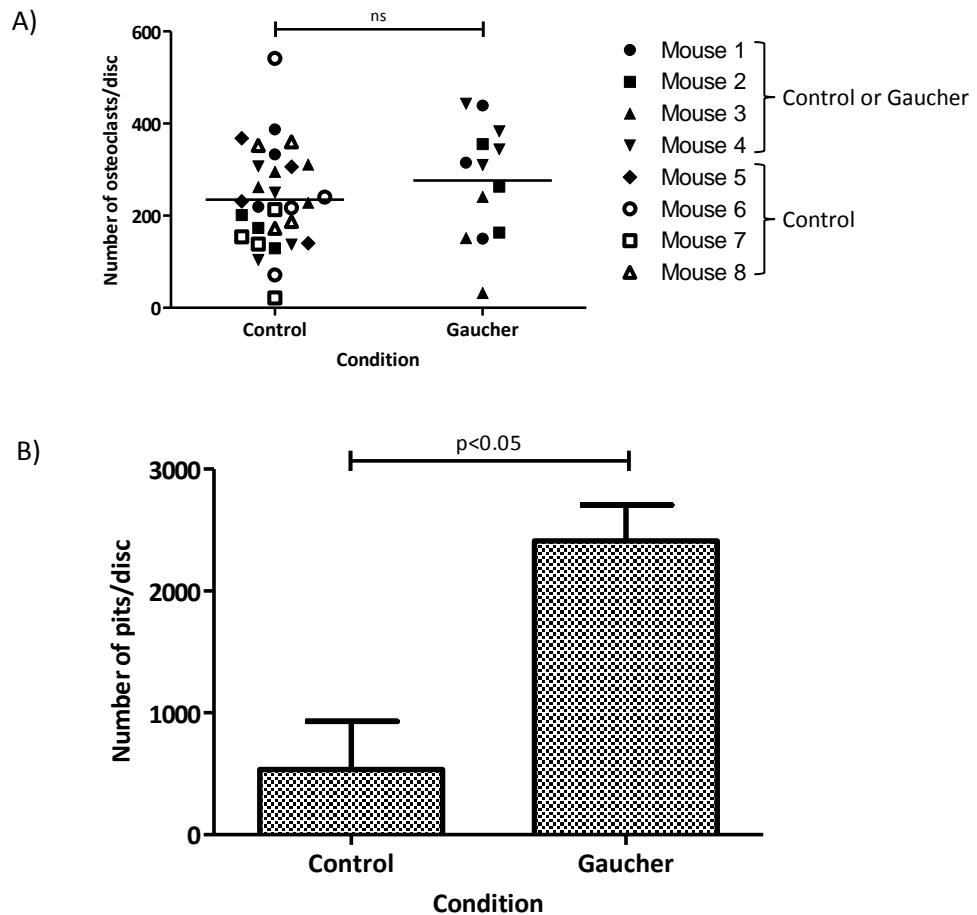


Figure 5.5 *in vitro* osteoclast generation in $GBA^{-/-}$ mice and healthy controls

Bone marrow was harvested from $GBA^{-/-}$ mice and their WT siblings at the time of sacrifice and used to generate osteoclasts *in vitro*. The KO group had four mice and the control group contained eight. A) Osteoclast-like cells differentiated on glass coverslips were stained for TRAP activity and osteoclasts were defined as large, multinuclear cells with positive TRAP stain. There was no significant difference in the number of osteoclasts generated between the two conditions. B) Osteoclasts generated on dentine discs were lysed and the discs stained with toluidine blue to reveal resorption pits. Osteoclasts produced from KO bone marrow produced significantly more resorption pits indicating increased activity levels compared to the control cells. Significance was tested using an unpaired t test, ns=p>0.05.

5.3.4 Viral transduction of osteoclast cultures

One of the primary reasons for investigating the potential of gene therapy as a GD treatment is because the current therapy does not have a significant effect on the skeletal complications associated with the disease. It is hoped that gene therapy of patient derived HSCs could be an effective method of treating these complications. Having demonstrated evidence of an overactive phenotype in osteoclasts generated from bone marrow of the *GBA*^{-/-} mouse, the next step would be to transduce the differentiating osteoclasts with a *GBA* vector and assay for correction of the phenotype.

First it was necessary to determine what point in the culture is optimal to perform the transduction. Bone marrow was harvested from a WT mouse and osteoclast cultures were set up as for the *GBA*^{-/-} mice but with the cells grown on glass coverslips as opposed to dentine discs. Viral transductions were performed on days one, two and three of the culture using an MOI of 10. When the cultures were terminated, the number of GFP positive cells was determined as an indication of which transduction gave the highest efficiency.

Transducing cells on day one (the day of harvest) gave a transduction efficiency of less than 38.5% (standard deviation: 11.96%). Transduction efficiency this low is not unexpected for this condition as the first day represents a selection step and therefore, not all of the cells transduced would be used in the final culture. Transducing the cells on day two or three of culture gave transduction efficiencies of 60.2% (standard deviation: 20.99%) and 71.5% (standard deviation: 0.12%) respectively but the culture transduced on day two showed much greater variation and therefore it would probably be optimal to transduce osteoclast differentiation cultures on day three. Unfortunately time did not allow the development of this work but it is an aspect which warrants further investigation.

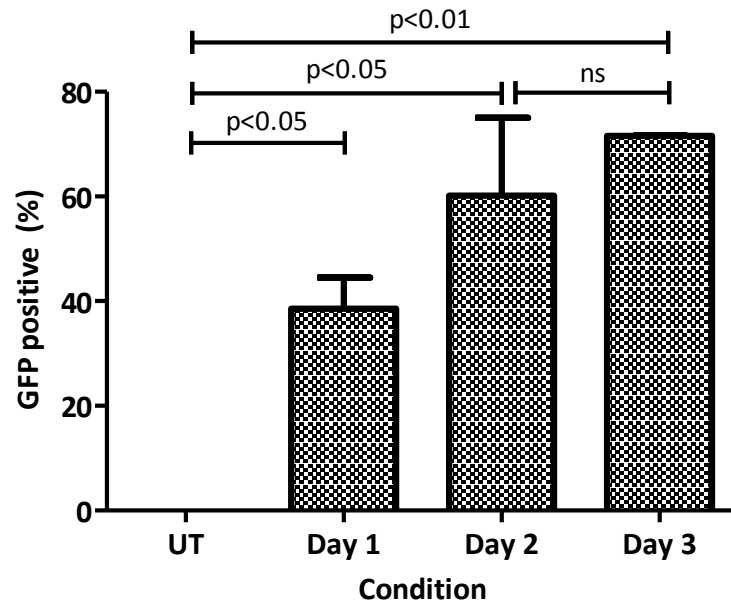


Figure 5.6 *in vitro* transduction is most effective when performed at day 3 of osteoclastogenesis.

Bone marrow was harvested from CD1 mice and differentiated into osteoclast-like cells *in vitro*. Transduction was performed with a vector carrying *eGFP* on each of the first three days in the protocol. After terminating differentiation and fixing the cells, the number of GFP positive cells in each culture was counted to assess the efficiency of transduction. The results showed that transduction is most effective when performed later in the protocol as the levels of GFP expression did not reach significance in cells transduced on the first day of differentiation. Error bars show standard deviation, $n=3$, significance was tested using one-way ANOVA with Tukey post-hoc test $ns=p>0.05$.

5.3.5 CFU-assay in transduced bone marrow

For the gene therapy strategy envisioned by this work to succeed it is vital to show that transducing *GBA*^{-/-} progenitors with a GC carrying vector will not result in toxicity to the stem cells as has been seen with the globoid cell leukodystrophy vector (391).

To investigate this, lineage negative cells isolated from the bone marrow of *GBA*^{-/-} mice were transduced with vectors carrying either *GBA* or *eGFP* at an MOI of 50. Murine lineage negative cells are those which do not bear any markers of haematopoietic differentiation and can therefore be assumed to be a population of immature progenitor cells. These cells were isolated from whole bone marrow using the microbead lineage depletion kit from Miltenyi Biotec. The transduced cells were then cultured in a semi-solid medium containing the necessary cytokines (stem cell factor, interleukin-3, interleukin-6, and erythropoietin) to stimulate haematopoietic differentiation. After 10 days of culture the number of colonies was assessed to determine if there was an effect on the viability or differentiation of cells transduced with the *GBA* vector (Figure 5.7). These data show that there is no significant difference between any of the conditions which suggests that the vector does not have a toxic effect on HSCs. However this work was performed on a relatively small number of mice and the inherent variability of such protocols, as indicated by the large error bars, combined with the fact that the results do show a downward trend after transduction with the *GBA* vector means that further investigation with a greater number of replicates is required before any firm conclusions can be drawn.

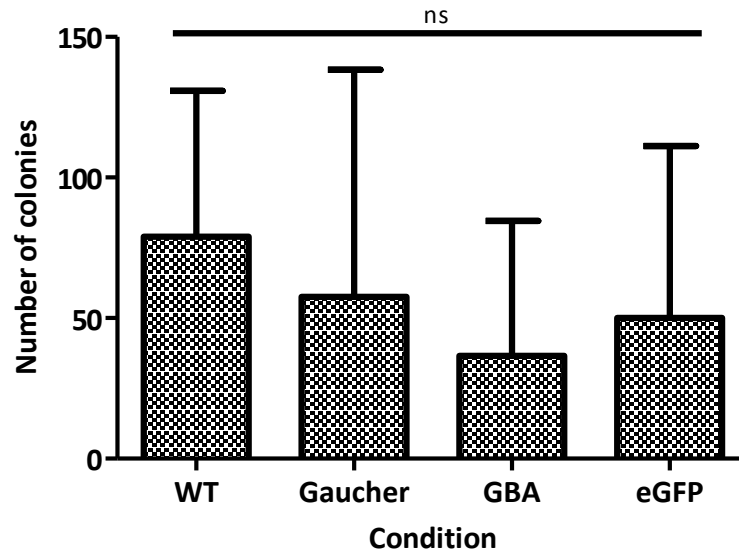


Figure 5.7 Colony forming assay with transduced $GBA^{-/-}$ bone marrow

Bone marrow harvested from three $GBA^{-/-}$ mice and three of their WT siblings (control group) was cultured in semi-solid medium for 10 days to encourage the formation of haematopoietic colonies. The results suggest that there is no significant difference in the number of colonies produced by $GBA^{-/-}$ mice even when the marrow had been transduced with either GBA or $eGFP$ lentiviral vectors. Error bars show standard deviation, significance was measured with 1-way ANOVA using Bonferroni's post-hoc test and with $p < 0.05$.

5.4 Summary

Bone disease is the main feature of type I GD that remains refractory to enzyme replacement therapy, and the results of our survey confirm that it is still a major aspect of the disease, but it is one that may be alleviated by gene therapy.

The data in this chapter has confirmed that autologous bone marrow progenitors modified by gene therapy are capable of differentiating into osteoclasts both *in vitro* and *in vivo*. This is important as there have been very few other demonstrations of osteoclastogenesis after gene therapy in the literature. One paper that has done so transduced CD34+ cells from patients with infantile malignant osteopetrosis and differentiated them *in vitro* to produce osteoclasts with restored functionality. However they were unable to confirm that osteoclastogenesis also occurs *in vivo* due to the species specificity of M-CSF (377). In this project we have been able to show the differentiation of osteoclasts from modified bone marrow both *in vitro* and *in vivo*.

We have also demonstrated that a mouse model of type II GD has a previously unknown osteoclast phenotype. It has not been possible to observe this phenotype in the living mice because they die at the age of two weeks (183, 184) which is before the point at which bone disease would likely develop. However by harvesting bone marrow and performing the differentiation *in vitro* we were able to show that, while the same number of osteoclasts differentiate from the bone marrow, they are more active and there is an increased level of bone resorption. Currently the reason for the skeletal disease in humans is unknown although it is thought to involve a disruption of the balance between osteoclast and osteoblast action with a potential role for both cell types. Therefore it would be of interest to follow up this work with an investigation of osteoblasts from the model mouse to compare their activity to those of wild type mice. Such an experiment would help indicate whether treating the osteoclast in the manner

suggested here would be likely to ameliorate bone disease or whether it would be beneficial to attempt to develop an osteoblast directed therapy.

This work has shown that, despite death occurring at an age before bone disease develops, the type II GD mouse model can still serve as a useful model for the osteoclast phenotype found in GD and to demonstrate further evidence for the involvement of the osteoclast in GD bone pathology. It has also demonstrated that gene therapy can result in genetically marked osteoclasts which strengthens the hypothesis that gene therapy of GD would ameliorate the skeletal symptoms of the disorder in a way that is not possible with ERT.

6 Discussion

Gaucher disease is haematopoietic in origin and leaves patients unable to process glucocerebrosides causing their accumulation within the lysosomes of phagocytic cells (4, 13, 14). Affected individuals experience hepatosplenomegaly, pancytopenia and skeletal complications (53, 56, 74) as well as neurological deterioration in the most severe subtypes (51, 65). Currently the majority of patients are treated with enzyme replacement therapy using preparations of recombinant or gene-activated enzyme however given the high cost of the treatment and the fact that it does not provide a permanent cure, there is room to consider other options.

One such option may be gene therapy which was first hypothesised over 40 years ago (196) but which has experienced a number of stumbling blocks throughout its history. Nevertheless recent years have seen an ever increasing number of clinical trials showing significant therapeutic benefit in a wide range of conditions including haemophilia (392), Parkinson's disease (393) and Leber congenital amaurosis, a form of inherited blindness (394). Most importantly in 2013 the first gene therapy was licensed for sale in the EU (395) for the treatment of lipoprotein lipase deficiency (396).

Many early studies employed γ -retrovirus-based vectors because of their innate ability to integrate foreign DNA into the host chromosome, resulting in permanent correction of transduced cells (259, 260). However the publication of data linking integration of γ -retroviral genomes close to proto-oncogenes with the development of leukaemia (234-236) led to a shift in vector usage.

Lentiviral vectors are derived from a family of the Retroviridae including HIV-1 and other immunodeficiency viruses. They retain the classic retroviral ability to integrate into the

host genome but appear to have an altered integration profile (261) which greatly reduces the association with oncogenesis (397-399).

Integration of the retroviral genome was believed to occur at random sites in the host chromosome until it was shown that different virus types show a preference for particular types of DNA sequence. The γ -retrovirus, such as murine leukaemia virus (MLV) shows a distinct preference for integrating at the start site of genes (262, 263). This helps explain the high rate of insertional mutagenesis after transduction with a γ -retroviral vector as proximity to a transcription start site can increase the effect of the viral enhancers to upregulate the neighbouring gene. The integration profile of lentiviruses is different as they show a tendency to integrate within genes meaning that there is less opportunity for interactions with the regulatory regions of other, nearby genes (261-263).

A crucial experiment performed in the tumour-prone Cdkn2a mouse strain used HSCs transduced with either lentiviral or γ -retroviral vectors carrying the eGFP gene to compare the genotoxicity of the two vector types (398). The work showed that while there was no difference in the tumour-free survival rates of mice in the different conditions, tumours in γ -retroviral treated mice had a much lower vector copy number than those of mice who received the lentivirus treated HSCs. Integration site analysis of the tumours revealed a significantly higher proportion of integration events within 10kb of the transcription start site in the γ -retroviral group but no such bias within lentiviral tumours which correlates with the previous *in vitro* findings on integration site preference and oncogenesis. This study indicated that a γ -retroviral integration event has a greater chance of leading to oncogenesis than that of a lentiviral genome (398).

It is not only the integration profile of lentiviruses which makes them more attractive than γ -retroviruses. The genome of γ -retroviruses is only able to integrate into the host chromosomes of cells during cell division as the genome is unable to cross the nuclear

membrane (400, 401). This is not the case with lentiviral genomes which are able to integrate while the nuclear membrane is intact and therefore can efficiently transduce non-dividing cells such as those in post-mitotic tissues and stem cells which divide at a very slow rate (400, 402).

Gene therapy is an attractive option for GD as lentiviral correction of the haematopoietic stem cell (HSC) should result in permanent correction of the haematopoietic lineage removing the requirement for macrophage directed ERT.

A number of studies have been performed previous to this project which have demonstrated the feasibility of using gene therapy to correct the enzymatic defect in GD (209, 363-366, 370, 371) but the majority of these used γ -retroviral vectors which are less relevant than those derived from lentiviruses due to the low rate of stem cell transduction.

A clinical trial in 1998 failed to show any efficacy of gene therapy when used to treat three GD patients but this is likely to be because the protocol did not include a conditioning step (373). A significant number of gene therapy protocols to date have been intended to treat primary immunodeficiencies in which the corrected stem cells have a selective advantage over the untreated HSCs within the bone marrow and therefore there is no requirement for conditioning to destroy the existing stem cell component. However, this is not the case in GD which does not have an immunodeficiency associated with it and therefore it is likely that a successful gene therapy treatment strategy will include myeloablative conditioning before the transfusion of corrected HSCs (Adrian Thrasher, personal communication).

A number of LSDs and similar, metabolic disorders stand to benefit from the development of gene therapy as the majority of conditions are monogenic and arise from

enzyme deficiencies in the haematopoietic compartment. Notably, gene therapy is currently in clinical trials for the treatment of adrenoleukodystrophy (ALD) and metachromatic leukodystrophy (MLD). The ALD trial treated two patients who were already showing the progressive demyelination of the CNS associated with the disease. Both patients were treated with autologous HSCs which had been transduced with a lentiviral vector carrying the ABCD1 gene and follow up has been reported for over three years. In common with patients who have undergone allogeneic HSCT, the gene therapy patients showed continuation of demyelination followed by stabilisation and some reversion of the disease (397, 403). The period of continued demyelination occurs after HSCT because stabilisation of the condition relies on repopulation of the CNS by donor-derived microglia and this can take up to 24 months (404) although studies in mice have shown that the process can be augmented by extensive conditioning which will fully ablate the haematopoietic cells of the CNS (405). The same strategy was used in a trial for MLD and showed stabilisation of disease up to 24 months post-gene therapy with treated patients remaining asymptomatic with arrested demyelination and continued neurological development (406).

Both of these studies have demonstrated the potential for gene therapy in metabolic disease and the promising results achieved with the CNS symptoms of both disorders allow for speculation that a gene therapy strategy could also be of benefit to type II and III GD patients. Both type II and III GD cause progressive neurological deterioration which is ultimately fatal. Despite being far from conclusive, data from type III HSCT recipients has suggested that the procedure can bring about a stabilisation of the neurological disease (71, 101, 407) and so it is conceivable that these patients may also benefit from HSC-directed gene therapy.

However the situation with type II GD is very different. CNS pathology likely develops *in utero* and is almost always in evidence at diagnosis (51, 408). Therefore it is unlikely that any intervention will be able to restore the damage which has already been done and so type II patients are not expected to benefit from the style of gene therapy used in this work (408, 409).

This project produced lentiviral vectors which could be used for the gene therapy of Gaucher disease. The vectors carry a WT glucocerebrosidase gene which had been cloned from healthy tissue during previous work (Ahad Rahim, unpublished).

Transduction of both HEK293T and GD patient-derived fibroblasts cell lines with the *GBA* vector resulted in a significant increase in both the expression and activity levels of the GC enzyme. The results of a time course study looking at expression levels in the first two weeks after transduction revealed a peak in activity after three days which then dropped but remained at a level 13-fold greater than that of untreated cells. These results confirmed that the vector produced is able to mediate expression of functional GC enzyme and can correct the defect in GD tissues. The reduction in activity three days post-transduction is not unexpected and is potentially due to the clearance of non-integrated viral genomes from the cell. This clearance occurs as part of innate cellular defence mechanisms to remove any non-integrated linear DNA which may be of viral origin and thereby protect the cell from potential infections (410, 411).

The vectors produced were under the control of a strong viral promoter (spleen focus-forming virus: SFFV) which is not commonly used in clinical vectors as a high proportion of viral elements may allow for recombination with endogenous retroviruses and produce replication competent viral particles. In addition to this, viral DNA sequences are often recognised as foreign by the host cell and so are likely to undergo methylation and other forms of regulation which can lead to silencing of the transgene especially when a

transduced stem or progenitor cell differentiates (340). The SFFV promoter was used in this study to provide reliable, high levels of protein expression which were required for this proof-of-concept work. To make the vectors produced in this project clinically applicable it would be necessary to exchange the SFFV promoter for a promoter sequence such as elongation factor 1 α (EF1 α) or phosphoglycerate kinase (PGK).

The SFFV promoter was used in a clinical trial for the treatment of X-linked chronic granulomatous disease (CGD) in which two patients received transduced autologous HSCs (320). Both patients showed clinical resolution of disease after treatment with the clearance of pre-existing infection in addition to correction of the molecular defect. Unfortunately, both patients showed clonal expansions of transduced cells which ultimately led to the development of myelodysplastic syndrome and the death of one patient (412). However that study used a γ -retroviral vector and so is not comparable to the system being used here. In contrast a trial for the gene therapy of ADA-SCID using a lentiviral vector carrying the transgene under the control of the SFFV promoter has now treated 5 patients without the development of any monoclonal expansions suggesting that the promoter itself is not overtly oncogenic when used with a lentivirus (413, 414).

Rather than using a viral promoter which may be subject to methylation or involved in the induction of mutagenesis, many researchers prefer to use a mammalian promoter element and two of the most popular are the EF1 α and PGK elements. EF1 α is a housekeeping gene and the promoter has been shown to give high levels of protein expression which is maintained over a longer time period than viral promoters (415, 416).

A further option when considering primer choice is to use a promoter which is restricted to the target cell or tissue which for GD is the macrophage. Levin *et al.* (2012) compared five macrophage specific promoters, three variations of the CD68 and two forms of a synthetic promoter (described in (417)), with the SFFV promoter (418). The results

showed that both promoters expressed the transgene at lower levels than SFFV and that although expression in non-macrophage cells was reduced with the non-viral promoters it was not completely abolished especially when the multiplicity of infection was high. For GD, restriction of transgene expression to the macrophage is not required as GC is a housekeeping gene (378-380) and therefore it is not necessary to prevent ubiquitous expression.

However, something that may require further investigation is the effect of expressing GC at high levels in the stem cell. Visigalli *et al.* (2010) showed that expression of galactosylceramidase (GALC) in the HSC of Krabbe disease mice led to rapid death of the transduced cells due to the sudden increase of the pro-apoptotic molecule ceramide from digestion of the stored sphingolipid (419). To prevent this cell death the researchers found it necessary to fuse a target sequence for an HSC-specific miRNA to the transgene to prevent its transcription within the HSC. As glucocerebroside is also a sphingolipid whose metabolism results in the production of ceramide the same problem could occur in transduced GD HSCs.

In Figure 5.7 of this project, bone marrow from a GD model mouse was transduced with the vectors and cultured in semi-solid medium to promote the differentiation of haematopoietic cells. No significant differences were found in the numbers of differentiated colonies produced by transduced or untransduced cultures but there was a high degree of variation within the groups which suggests that, with further investigation, transduction may be found to affect survival and differentiation of HSCs. If this is shown to be the case the use of miRNA or a promoter which is not expressed within the HSC may be necessary to restrict *GBA* expression.

Although the main symptoms of GD are clearly associated with the phagocytic cells of the haematopoietic system, *GBA* is in fact a housekeeping gene and is expressed ubiquitously, albeit at low levels (378-380, 389). There is no evidence to suggest that other cells are compromised by the lack of *GBA* expression, but it may prove to be beneficial if it is possible to correct cells of other lineages in addition to haematopoietic cells. We therefore examined strategies that might allow for non-genetic correction of cells outside the haematopoietic system.

It is known that the native *GBA* sequence contains a secretion signal although it is not considered to be a secreted protein as the enzyme is delivered directly to the lysosome from the endoplasmic reticulum by association with lysosomal integral membrane protein 2 (LIMP2) (23, 24). However, it is possible that this secretion signal may be used when the protein is overexpressed in order to prevent the accumulation of enzyme within a cell. Experiments shown here have confirmed that this is the case; when expressed at high levels within a cell, excess GC is secreted into the extracellular environment. It is also shown that secreted protein retains its enzymatic activity. This work demonstrates that transduced cells which are overexpressing the transgene could act as reservoirs of protein with secreted enzyme potentially being available for use by untransduced cells.

The secretion of GC after transduction by a *GBA* carrying virus has been demonstrated before from a number of cell types including GD fibroblasts (388) as well as both human and murine myoblasts (420). These studies were also able to show uptake of the secreted enzyme by untransduced GD fibroblasts (388) or WT murine macrophages (420). The uptake of GC by macrophages occurred at a much higher rate than uptake by either the fibroblasts in Liu *et al.* (2004) or the LCLs used in this study which perhaps reflects the higher requirement of that cell type for the enzyme or the presence of the mannose

receptor which is the native receptor for the enzyme. However it is uptake by the fibroblast which is most relevant to this study; uptake by non-haematopoietic cells such as fibroblasts will be crucial for the success of the cross-correction strategy suggested here. The uptake by fibroblasts in the study by Liu *et al.* was only modest but as that work used the native enzyme, it is hoped that the addition of a PTD will improve levels of uptake.

The protein used for ERT is deliberately targeted to macrophages by the exposure of mannose residues on the glycoprotein side chains allowing for uptake by the mannose receptor (109). However this is a macrophage specific receptor and therefore an alternative strategy is required to target other cell types.

The method we investigated for engineering uptake of secreted protein by neighbouring cells was to fuse a protein transduction domain (PTD) to the native protein sequence. We chose to use the PTD which has been isolated from the HIV-1 TAT protein because it has been shown to work independently of receptor and cell type (346, 347). Previous studies have shown that the TAT PTD can vastly improve the biodistribution of exogenous protein in an ERT style setting (421).

Lee *et al.* (2005) fused the TAT PTD to GC in an attempt to improve uptake of the enzyme used for ERT (315). They found that fusion of the PTD to the C-terminus of the protein greatly increased the levels of enzyme taken up by GD fibroblast when compared to both imiglucerase and the native protein. However they also found that adding the PTD to the N-terminus of GC abolished protein expression. Further investigation of the PTD sequence by a group at King's College London identified two potential furin endonuclease cleavage sites which could explain the absence of protein produced from the N-terminal fusion protein (316) if recognition of the cleavage sites leads to degradation of GC. The King's College researchers were able to abolish these cleavage sites by altering the

nucleotide sequence without affecting the transduction capability of the domain (mTAT) and with this modified TAT sequence we were able to produce an N-terminal fusion protein which expresses at comparable levels to the C-terminal constructs. This seems to support our hypothesis that in the study by Lee *et al.*, the N-terminal fusion construct produced is expressed but that recognition of the furin cleavage sites leads to degradation of the enzyme and that this effect is abolished by modifying the TAT sequence.

In this study GC was fused to either the mTAT or the WT TAT version of the PTD and assayed for their effect on uptake of secreted enzyme by untransduced lymphoblastic cells. When they were co-cultured with fibroblasts expressing either version of the TAT domain fused to the C-terminus of the protein there was a seven-fold increase in GC enzyme activity within the untransduced cells compared to a modest doubling of enzyme activity in cells in the N-terminal fusion condition. The failure of the N-terminal construct to mediate high levels of protein transduction could be due to the way the protein folds during assembly. The transduction domain must be on the exterior of the protein after assembly in order to be accessible to the cell membrane; if it is obscured it may not be able to mediate transduction (346, 347).

This work used transduced fibroblasts as the reservoir of protein and untransduced, GD patient-derived LCLs as the test cell line for uptake of secreted protein. In reality this is the reverse of the situation that would be seen in patients. The gene therapy strategy suggested by this work would transduce cells of the hematopoietic system and it would be these cells which secrete the protein allowing for the cross-correction of other tissues. Therefore, although this work demonstrates that cross-correction is a valid theory and that addition of a PTD can be used to enhance the effect, it does not accurately reflect the situation which would occur in a patient and further work would need to focus on

recapitulating the *in vivo* scenario to demonstrate that cross-correction is a suitable strategy for use in GD.

A number of previous studies have shown that the TAT PTD can be used to improve the biodistribution of exogenous proteins in whole animals. For example, Grubb *et al.* (2010) showed that fusion of the TAT PTD to β -glucuronidase (GUS), the enzyme deficient in mucopolysaccharidosis (MPS) VII, resulted in an enzymatic preparation which greatly increased the degree of substrate clearance from the renal cells of MPS VII mice (317). The authors also reported seeing clearance of stored material from osteoclasts and osteoblasts which is of great significance for this work as it suggests that the TAT PTD is capable of mediating protein transduction into osteological cells, something we aim to achieve with our treatment strategy.

In addition to potentially transducing the osteoblast with the transprotein, it may be possible to target neurological symptoms of type III GD. Farfel-Becker *et al.* (2014) showed that accumulation of GlcCer within neurons correlates with neurological deterioration *in vivo* (68). Another study by Pelled *et al.* (2000) showed that neuronal cultures which had been treated with a chemical inhibitor of GC could be prevented from undergoing apoptosis after culture with imiglucerase which may suggest that correction of the enzymatic defect may be able to mediate prevention or stabilisation of neurological disease (422). There has also been suggestion that fusion of the TAT PTD to a protein can allow the protein to cross the blood-brain barrier (357). If testing was to show that the TAT domain can stimulate or improve uptake of GC by neurons it may mean that the therapy suggested here could be extended to treatment of the neurological component of type III GD.

Although cross-correction of untransduced tissues may be of some benefit to patients, there is also a risk that secretion of the enzyme may lead to an immunological response

against the protein. An immune response against the transprotein could result in its clearance from the extracellular environment and therefore prevent cross-correction from occurring. Although there is evidence that long-term treatment with exogenous enzyme only produces an inhibitory immune response in a low number of GD patients, it is a significant problem in replacement therapy for other conditions including Pompe disease (423, 424) haemophilia A (425, 426) and Fabry disease (427, 428) so it is therefore something which must be considered.

With the introduction of ERT, many of the symptoms associated with GD have been significantly reduced and patients are able to live relatively symptom-free lives. However the one aspect of type I GD which remains prevalent is bone disease. Treating the skeletal complications of GD is further complicated as the exact cause of the symptoms remains unclear. However, mounting evidence has recently been described which suggests the involvement of an osteoclast phenotype. It has been shown that a number of cytokines involved in osteoclastogenesis are found at elevated levels in the plasma of GD patients (85, 90, 93, 94) although the levels of each cytokine tend to vary between patients. It is also worth considering that the majority of these factors are produced by the macrophage and so correction of the HSC should normalise levels and potentially alleviate the skeletal disease. More compelling evidence comes from Reed *et al.* (2013) who showed that, under the same culture conditions, precursor cells derived from GD patients are more likely than controls to differentiate into osteoclasts and that those osteoclasts are significantly more active than their counterparts which may help explain the bone pathology seen in patients (86).

Based on this work we hypothesised that a similar phenotype may be observable in osteoclasts derived from the HSCs of the *GBA*^{-/-} mouse used in this project. It was found

that while bone marrow harvested from knock-out mice did not undergo increased osteoclastogenesis as was the case with human cells, they did show the same hyperactivity of generated osteoclasts. This is a significant finding as very few of the GD mouse models which have been developed to date have shown a skeletal phenotype and little work has been done to discover whether any of them show a defect *in vitro*. This work therefore presents a new potential tool for the investigation of the skeletal phenotype of GD.

This finding is contrary to some previous findings including the skeletal phenotype found in the type I GD mouse model produced by Mistry *et al.* (2010). This work suggested that *GBA* deletion had no effect on the activity of osteoclasts but that the skeletal phenotype (including osteonecrosis and osteopenia) was due to impaired bone formation caused by reduced osteoblast formation (89). The work of Mucci *et al.* (2013) also supports the involvement of the osteoblast in the development of a skeletal phenotype as they found that chemical inhibition of GC in peritoneal macrophages led to reduced collagen deposition and mineralisation *in vitro* (80). However they also showed an increase in osteoclast formation and activity in their chemical *in vitro* model. Given all of this work it seems most likely that a combination of osteoclast and osteoblast effects lead to the development of skeletal symptoms in patients.

Gene therapy of the HSC should lead to direct correction of the *GBA*^{-/-} osteoclast and therefore alleviate any symptoms which are due to enzymatic defect in that cell but it may also be possible to reduce the effect of GD osteoblasts if the cross-correction strategy is successful. Mesenchymal stem cells and more committed osteoblast progenitors are found in the bone marrow (429, 430) and therefore will be exposed to GC secreted by the transduced HSCs which will also be in the bone marrow cavity. If the cells

are able to take up that secreted enzyme this correction could be capable of ameliorating the disease phenotype from both sides of bone homeostasis.

Knowing that a phenotype can be identified in *in vitro* derived, murine osteoclasts could be of substantial benefit to researchers working on gene therapy for GD. Transduction of precursor cells on day 2 or 3 of the differentiation protocol gives rise to a high number of transduced osteoclasts. This presents a measurable effect for future work investigating the principle of using gene therapy to correct the osteoclast defect.

Hematopoietic gene therapy has traditionally been used to treat blood disorders and there has been very little research into correcting skeletal conditions using HSCs. Where gene therapy has been suggested for bone disorders, it has generally involved treating more committed precursor cells.

However, a group in Sweden recently published a study which showed that it is possible to treat the osteoclast disorder osteopetrosis by genetically correcting HSCs using lentiviral vectors (377). We were also able to demonstrate that genetically modified HSCs are capable of differentiating into osteoclasts when transplanted into irradiated recipients. HSCs were treated with a GFP expressing vector and transplanted into HLA-identical recipient mice. Nine weeks post-transplant, mice were culled and it was found that GFP positive osteoclasts could be isolated from the macerated bones of recipient mice demonstrating not only that the transplanted HSCs could differentiate into osteoclasts, but also that the differentiated cells retained the ability to express the viral transgene. These two pieces of work taken together suggest that gene therapy of HSCs should be able to correct the skeletal phenotype associated with GD.

Further work is required to comprehensively show the extent to which the osteoclast is responsible for the skeletal complications. The osteoblast is a cell of mesenchymal origin

and therefore will not be directly affected by the gene therapy strategy suggested here. If the osteoblast is shown to be an important mediator in the development of skeletal complications another strategy may be required to target the skeletal symptoms of GD.

The appeal of gene therapy as a treatment method for Gaucher disease was reduced after the introduction of ERT but recently the inefficient resolution of skeletal symptoms and the global shortage of enzyme preparation have led to a resurgence of interest.

This project has shown that lentiviral transduction of patient cells can lead to correction of the metabolic defect in GD as well as producing a reservoir of protein which can be used to treat untransduced cells in a gene-independent fashion. The potential for treating the osteoclast in addition to cells of the haematopoietic system could be a significant bonus to gene therapy as compared to ERT but further work needs to be done to confirm the underlying defect and whether genetic correction could reverse the phenotype and the pre-existing damage.

7 Appendix

7.1 Papers presented

Oral presentations

1. **Lentiviral vectors for gene therapy of Gaucher disease** – European Joint Lab Meeting, Rotterdam 2011
2. **Developing lentiviral vectors for gene therapy of type I Gaucher disease** – European Working Group on Gaucher Disease, Paris 2012
3. **Gene therapy for metabolic disease** – UCL Gene and Cell Therapy MSc course, 2013 and 2014

Poster presentations

1. **Lentiviral vectors for gene therapy of type I Gaucher disease** – European Society of Gene Therapy, Brighton 2011
2. **Lentiviral vectors for gene therapy of type I Gaucher disease** – British Society of Gene Therapy, London 2012
3. **Lentiviral vectors for gene therapy of type I Gaucher disease** – British Society of Gene Therapy, London 2013. Poster prize winner.
4. **Lentiviral vectors for gene therapy of type I Gaucher disease** – European Society of Gene Therapy, Madrid 2013

Funding for travel was kindly provided by the following groups:

1. **Jim Seakins Memorial Travel Fund**, Institute of Child Health UCL
2. **European Working Group on Gaucher Disease**
3. **Susan Lewis Memorial Award**, UK Gaucher Association

7.2 Sequence alignment of *GBA* including mTAT or WT TAT fusion

[illegible]

		850		900
GBA	GCTGAGCACA AGTTACAGTT CTGGGCAGTG ACAGCTGAAA ATGAGCCTTC TGCTGGGCTG TTGAGTGGAT ACCCCTTCCA GTGCCTGGGC			
GBA-mTAT	GCTGAGCACA AGTTACAGTT CTGGGCAGTG ACAGCTGAAA ATGAGCCTTC TGCTGGGCTG TTGAGTGGAT ACCCCTTCCA GTGCCTGGGC			
GBA-WT_TAT	GCTGAGCACA AGTTACAGTT CTGGGCAGTG ACAGCTGAAA ATGAGCCTTC TGCTGGGCTG TTGAGTGGAT ACCCCTTCCA GTGCCTGGGC			
mTAT-GBA	GCTGAGCACA AGTTACAGTT CTGGGCAGTG ACAGCTGAAA ATGAGCCTTC TGCTGGGCTG TTGAGTGGAT ACCCCTTCCA GTGCCTGGGC			
		950		
GBA	TTCACCCCTG AACATCAGCG AGACTTCATT GCCCGTGACC TAGGTCCTAC CCTCGCCAAC AGTACTCACC ACAATGTCCG CCTACTCATG			
GBA-mTAT	TTCACCCCTG AACATCAGCG AGACTTCATT GCCCGTGACC TAGGTCCTAC CCTCGCCAAC AGTACTCACC ACAATGTCCG CCTACTCATG			
GBA-WT_TAT	TTCACCCCTG AACATCAGCG AGACTTCATT GCCCGTGACC TAGGTCCTAC CCTCGCCAAC AGTACTCACC ACAATGTCCG CCTACTCATG			
mTAT-GBA	TTCACCCCTG AACATCAGCG AGACTTCATT GCCCGTGACC TAGGTCCTAC CCTCGCCAAC AGTACTCACC ACAATGTCCG CCTACTCATG			
	1000		1050	
GBA	CTGGATGACC AACGCTTGCT GCTGCCCCAC TGGGCAAAGG TGGTACTGAC AGACCCAGAA GCAGCTAAAT ATGTTTCATGG CATTGCTGTA			
GBA-mTAT	CTGGATGACC AACGCTTGCT GCTGCCCCAC TGGGCAAAGG TGGTACTGAC AGACCCAGAA GCAGCTAAAT ATGTTTCATGG CATTGCTGTA			
GBA-WT_TAT	CTGGATGACC AACGCTTGCT GCTGCCCCAC TGGGCAAAGG TGGTACTGAC AGACCCAGAA GCAGCTAAAT ATGTTTCATGG CATTGCTGTA			
mTAT-GBA	CTGGATGACC AACGCTTGCT GCTGCCCCAC TGGGCAAAGG TGGTACTGAC AGACCCAGAA GCAGCTAAAT ATGTTTCATGG CATTGCTGTA			
	1100		1150	
GBA	CATTGGTACC TGGACTTTCT GGCTCCAGCC AAAGCCACCC TAGGGGAGAC ACACCGCCTG TTCCCCAACA CCATGCTCTT TGCCTCAGAG			
GBA-mTAT	CATTGGTACC TGGACTTTCT GGCTCCAGCC AAAGCCACCC TAGGGGAGAC ACACCGCCTG TTCCCCAACA CCATGCTCTT TGCCTCAGAG			
GBA-WT_TAT	CATTGGTACC TGGACTTTCT GGCTCCAGCC AAAGCCACCC TAGGGGAGAC ACACCGCCTG TTCCCCAACA CCATGCTCTT TGCCTCAGAG			
mTAT-GBA	CATTGGTACC TGGACTTTCT GGCTCCAGCC AAAGCCACCC TAGGGGAGAC ACACCGCCTG TTCCCCAACA CCATGCTCTT TGCCTCAGAG			
	1200		1250	
GBA	GCCTGTGTGG GCTCCAAGTT CTGGGAGCAG AGTGTGCGGC TAGGTCCTG GGATCGAGGG ATGCAGTACA GCCACAGCAT CATCACGAAC			
GBA-mTAT	GCCTGTGTGG GCTCCAAGTT CTGGGAGCAG AGTGTGCGGC TAGGTCCTG GGATCGAGGG ATGCAGTACA GCCACAGCAT CATCACGAAC			
GBA-WT_TAT	GCCTGTGTGG GCTCCAAGTT CTGGGAGCAG AGTGTGCGGC TAGGTCCTG GGATCGAGGG ATGCAGTACA GCCACAGCAT CATCACGAAC			
mTAT-GBA	GCCTGTGTGG GCTCCAAGTT CTGGGAGCAG AGTGTGCGGC TAGGTCCTG GGATCGAGGG ATGCAGTACA GCCACAGCAT CATCACGAAC			
	1300		1350	
GBA	CTCCTGTACC ATGTGGTCGG CTGGACCGAC TGGAACCTTG CCCTGAACCC CGAAGGAGGA CCCAATTGGG TGCCTAACTT TGTGACAGT			
GBA-mTAT	CTCCTGTACC ATGTGGTCGG CTGGACCGAC TGGAACCTTG CCCTGAACCC CGAAGGAGGA CCCAATTGGG TGCCTAACTT TGTGACAGT			
GBA-WT_TAT	CTCCTGTACC ATGTGGTCGG CTGGACCGAC TGGAACCTTG CCCTGAACCC CGAAGGAGGA CCCAATTGGG TGCCTAACTT TGTGACAGT			
mTAT-GBA	CTCCTGTACC ATGTGGTCGG CTGGACCGAC TGGAACCTTG CCCTGAACCC CGAAGGAGGA CCCAATTGGG TGCCTAACTT TGTGACAGT			
	1351		1400	
GBA	CCCATCATTG TAGACATCAC CAAGGACACG TTTTACAAAC AGCCCATGTT CTACCACCTT GGCCACTTCA GCAAGTTCAT TCCTGAGGGC			
GBA-mTAT	CCCATCATTG TAGACATCAC CAAGGACACG TTTTACAAAC AGCCCATGTT CTACCACCTT GGCCACTTCA GCAAGTTCAT TCCTGAGGGC			
GBA-WT_TAT	CCCATCATTG TAGACATCAC CAAGGACACG TTTTACAAAC AGCCCATGTT CTACCACCTT GGCCACTTCA GCAAGTTCAT TCCTGAGGGC			
mTAT-GBA	CCCATCATTG TAGACATCAC CAAGGACACG TTTTACAAAC AGCCCATGTT CTACCACCTT GGCCACTTCA GCAAGTTCAT TCCTGAGGGC			
	1450		1500	
GBA	TCCCAGAGAG TGGGGCTGGT TGCCAGTCAG AAGAACGACC TGGACGCAGT GGCAGTGATG CATCCCAGATG GCTCTGCTGT TGTGGTCGTG			
GBA-mTAT	TCCCAGAGAG TGGGGCTGGT TGCCAGTCAG AAGAACGACC TGGACGCAGT GGCAGTGATG CATCCCAGATG GCTCTGCTGT TGTGGTCGTG			
GBA-WT_TAT	TCCCAGAGAG TGGGGCTGGT TGCCAGTCAG AAGAACGACC TGGACGCAGT GGCAGTGATG CATCCCAGATG GCTCTGCTGT TGTGGTCGTG			
mTAT-GBA	TCCCAGAGAG TGGGGCTGGT TGCCAGTCAG AAGAACGACC TGGACGCAGT GGCAGTGATG CATCCCAGATG GCTCTGCTGT TGTGGTCGTG			
	1550		1600	
GBA	CTAAACCGCT CCTCTAAGGA TGTGCCTCTT ACCATCAAGG ATCCTGCTGT GGGCTTCCTG GAGACAATCT CACCTGGCTA CTCATTAC			
GBA-mTAT	CTAAACCGCT CCTCTAAGGA TGTGCCTCTT ACCATCAAGG ATCCTGCTGT GGGCTTCCTG GAGACAATCT CACCTGGCTA CTCATTAC			
GBA-WT_TAT	CTAAACCGCT CCTCTAAGGA TGTGCCTCTT ACCATCAAGG ATCCTGCTGT GGGCTTCCTG GAGACAATCT CACCTGGCTA CTCATTAC			
mTAT-GBA	CTAAACCGCT CCTCTAAGGA TGTGCCTCTT ACCATCAAGG ATCCTGCTGT GGGCTTCCTG GAGACAATCT CACCTGGCTA CTCATTAC			
	1650		1677	
GBA	ACCTACCTGT GCGTCGCCA GTGA.....			
GBA-mTAT	ACCTACCTGT GCGTCGCCA GTACGCCAGA AAGGCCGCCA GGCAGGCCAG GGCATGA			
GBA-WT_TAT	ACCTACCTGT GCGTCGCCA GTACGGCCGC AAGAAGGCC GCCAGCGCCG CCGCTGA			
mTAT-GBA	ACCTACCTGT GCGTCGCCA GTGA.....			

Sequence alignments of the *GBA* constructs used in this project.

The wild type or modified TAT sequences are shown in red, leader sequence (including secretion signal) is highlighted in green and initiator codons are underlined.

7.3 Gene therapy factsheet sent to Gaucher patients

Gene Therapy: what is it and why is it worth talking about?

My name is Kathy and I'm a PhD student working at University College London (UCL). My project is looking at possible methods of developing gene therapy for type I Gaucher disease and so I am writing to you to ask for your perspective on the concept of gene therapy and whether it is something you as a patient would be interested in.

This article has been written to introduce you to the concept of gene therapy and how it can be applied to Gaucher disease.

- Gaucher disease is a genetic disorder in which patients have two non-functional copies of the *glucocerebrosidase* (*GBA*) gene.
- Gene therapy aims to correct this by inserting an additional, working copy of the *GBA* gene into the patient's own cells.

Therefore gene therapy has been suggested as a new method of treating Gaucher disease as the introduced gene could be able to compensate for the two non-functional copies the patient has.

What is it?

Gene therapy describes a treatment option where a genetic defect is corrected by addition of a healthy copy of the gene to a patient's cells.

How is it done?

We have chosen to use a lentivirus to correct the patient's own bone marrow stem cells in order to treat Gaucher disease.

- Lentivirus is a term that refers to a family of viruses which includes HIV. We use safe, disabled versions of these viruses for gene therapy because they have evolved over thousands of years to be very efficient at getting genes into cells.
- The virus we will use has had all of the genes used for replication and disease removed so that it is simply a harmless tool, like a case for delivering genes.
- Bone marrow stem cells are the foundation of the whole blood system; over the course of your life they divide and then differentiate to produce every type of blood cell in the body. This includes the macrophage which is the major cause of illness in Gaucher disease.

By using lentivirus which can effectively target stem cells and integrate the gene into the patient's chromosomes, we are able to get correction of the whole system as the gene will be passed on to both new cells when they divide. In this way it is possible that the blood could be permanently corrected with no need for enzyme replacement therapy (ERT).

Why would this work for Gaucher disease?

- Gaucher disease is caused by a mutation in a single, well-understood gene that makes the enzyme – glucocerebrosidase – which we have been able to insert into a virus.
- Bone marrow transplant from a healthy, matched donor has been shown in the past to cure type I Gaucher patients, which demonstrates that correcting the bone marrow can alleviate illness.

Initially this is being presented as a potential treatment option for type I Gaucher disease however there is also some evidence that after bone marrow transplant some of the stem cells differentiate into microglia; macrophage-like cells in the brain. If this is the case then it is possible that this treatment could lessen some of the neurological symptoms experienced by type III patients.

What would the procedure involve?

The procedure is very similar to bone marrow transplant but there is no need to find a matched donor as the patient's own bone marrow is used.

- To begin with bone marrow stem cells are harvested either from the bones, or from the blood (leukapheresis).
- To harvest bone marrow from the bones, the patient is put under general anaesthetic and a needle is used to remove cells from the pelvic bone. The procedure takes around one hour.
- For leukapheresis the patient is given a drug called G-CSF (granulocyte colony stimulating factor) over 5 days to encourage the stem cells to enter the blood stream. Blood is taken and used to isolate the stem cells.
- The cells are then grown in the laboratory for up to one week and a lentivirus is used to insert a functioning copy of the glucocerebrosidase gene. Some cells will be frozen for use in the unlikely event that the patient's bone marrow fails to recover after gene therapy.
- During this time the patient will undergo a course of chemotherapy to destroy any remaining stem cells in the bone marrow and allow the modified cells to be the dominant population after transplant. Without this 'conditioning' it is likely that the transplanted cells will struggle to survive as the existing cells will compete with them in the bone marrow space. This is usually the same treatment a patient receives before bone marrow transplant.
- The modified stem cells are then transplanted back into the patient in the form of an intravenous transfusion which will take around 30-45 minutes and the introduced cells should go on to form a complete, healthy blood system.
- The patient would have to remain in hospital while the immune system regenerates which is likely to be a period of one month to six weeks.

What are the potential benefits?

- It is a one-off procedure; targeting the stem cell and integration of the gene into the chromosomes could give permanent correction of the blood system removing the need for further treatment.
- Because this treatment uses the patient's own bone marrow there is much lower risk of rejection than with a conventional bone marrow transplant.
- This method should also correct an important bone cell – the osteoclast – and so may be able to reduce or prevent the bone pain and other skeletal symptoms associated with type I Gaucher disease.
- The protocol performs all the genetic manipulation outside of the body so it is very unlikely that the gene would be inserted into any other cells in the body.

What are the drawbacks?

- The virus inserts the gene into the patient's DNA at a random site so there is a very low risk of a phenomenon known as insertional mutagenesis. This occurs when the gene integrates close to, and affects the function of, another gene. In a small number of cases where gene therapy using an early version of another virus was successful in treating an immune disorder, insertional mutagenesis led to some patients developing a form of leukaemia. However, this is rare and has never been seen with the kind of virus we are proposing for treating Gaucher disease.
- The chemotherapy used can cause a number of side effects including nausea and temporary hair loss. In particular it used to destroy the immune system and so leaves the patient at increased risk of contracting infections until the immune system has been restored by the transplanted cells. There is also a risk of chemotherapy leading to infertility.

- This treatment is experimental and may not work, but if gene therapy is unsuccessful the patient would be able to resume enzyme replacement therapy.

Has it been done before?

Yes! Gene therapy has been successfully used to treat a range of disorders.

Problems with the immune system such as:

- X-linked severe combined immunodeficiency
- Adenosine deaminase deficiency
- Chronic granulomatous disease
- Wiskott-Aldrich Syndrome

Other diseases including:

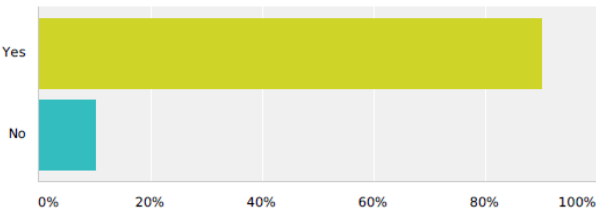
- Adrenoleukodystrophy; a disorder which affects the brain and featured in the film *Lorenzo's Oil*
- A form of blindness called Leber congenital amaurosis (LCA)
- Haemophilia B; a blood clotting disorder

Gene therapy has been suggested as a treatment option for Gaucher disease before and a clinical trial involving three patients was run in 1998. However this trial did not produce a prolonged improvement in any of the treated patients, probably because the transplanted cells failed to establish themselves in the bone marrow. This trial also used a different type of virus to the one we are using in our current work. It did however show that the procedure used is safe as the patients didn't experience any adverse events and treated patients were able to resume ERT at the same dose they had previously taken.

7.4 Results of survey to Gaucher patients

Q1 Are you a patient

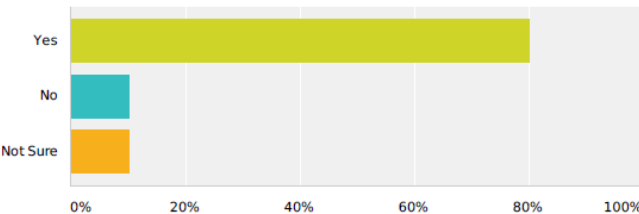
Answered: 10 Skipped: 0



Answer Choices	Responses
Yes	90%9
No	10%1
Total	10

Q2 Before reading this fact sheet, were you aware of gene therapy?

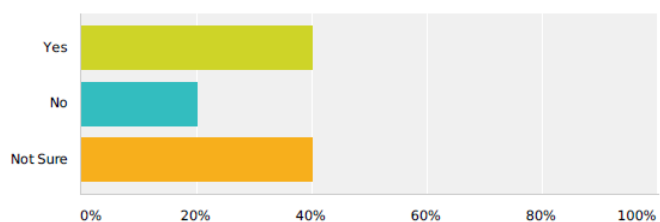
Answered: 10 Skipped: 0



Answer Choices	Responses
Yes	80%8
No	10%1
Not Sure	10%1
Total Respondents: 10	

Q3 If yes to Q1, would you have considered it as an option for Gaucher disease?

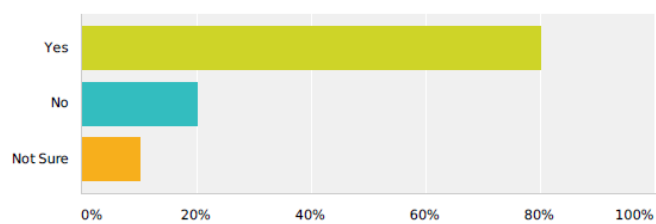
Answered: 10 Skipped: 0



Answer Choices	Responses	
Yes	40%	4
No	20%	2
Not Sure	40%	4
Total Respondents: 10		

Q4 After reading this fact sheet would you consider gene therapy as an option for Gaucher disease?

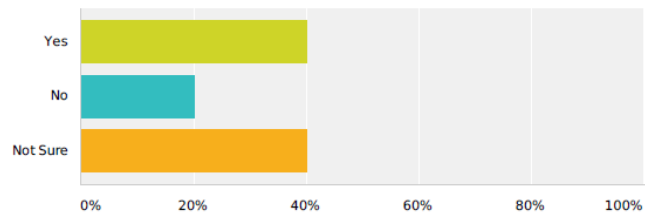
Answered: 10 Skipped: 0



Answer Choices	Responses	
Yes	80%	8
No	20%	2
Not Sure	10%	1
Total Respondents: 10		

Q5 If you or your child were given the opportunity to participate in a gene therapy trial (knowing that ERT would always be available as a back-up) would you be interested in doing so?

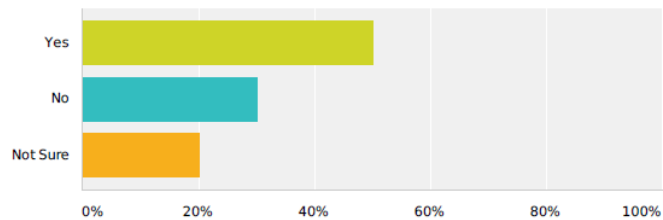
Answered: 10 Skipped: 0



Answer Choices	Responses	
Yes	40%	4
No	20%	2
Not Sure	40%	4
Total Respondents: 10		

Q6 If gene therapy was offered to you as a fully approved treatment option (once it has passed through trial stages) for you or your child, do you think it would be a preferable option to ERT?

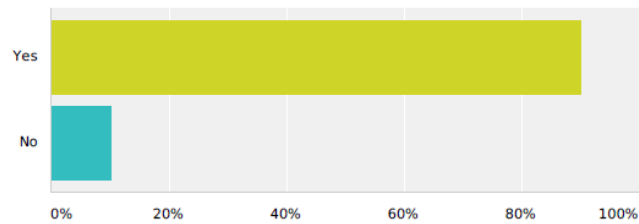
Answered: 10 Skipped: 0



Answer Choices	Responses	
Yes	50%	5
No	30%	3
Not Sure	20%	2
Total Respondents: 10		

Q7 Do you feel this fact sheet contains sufficient information to answer these questions?

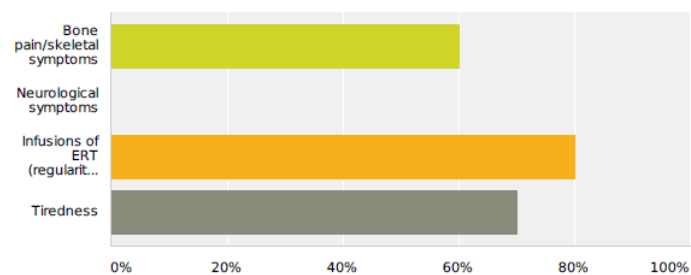
Answered: 10 Skipped: 0



Answer Choices	Responses
Yes	90% 9
No	10% 1
Total Respondents: 10	

Q8 We would like to know what, in your opinion and experience, are the most difficult aspects of Gaucher disease to live with:

Answered: 10 Skipped: 0



Answer Choices	Responses
Bone pain/skeletal symptoms	60% 6
Neurological symptoms	0% 0
Infusions of ERT (regularity/procedure)	80% 8
Tiredness	70% 7
Total Respondents: 10	

7.5 Free text answers to Q5

Q5: If you or your child were given the opportunity to participate in a gene therapy trial (knowing that ERT would always be available as a back-up) would you be interested in doing so?

- Yes - To eradicate the disease totally. Advancement in gene therapy is the long term answer to a disease that is very debilitating and enzyme treatment does not always prevent the disease from becoming more difficult
- Not sure - I would be willing to participate but would worry about the side effects or the possibility of things going wrong
- Not sure - Would have to think very hard about it given the intrusive nature of the trial, requirement for chemo, long period in hospital etc.
- No - am happy with enzyme replacement, it works and has limited side effects. (as type 1 patient I'm happy with enzyme replacement, I'm sure that gene therapy would have further benefits for type 3 patients if it was found to help the neurological aspect of condition.)
- Not sure - I have been on a medical trial before which me very unwell. (Q6. Offered fully licensed treatment would you take it? No.)
- Yes - I am in my mid 60s have had a family - lost most of my hair anyway and - if I became a GMO and was banned from Safeway - 'it wouldn't bother me' :-). Have been on ERT since 1996 and provided risk assessment seemed acceptable I believe contributing to such progress/research would help others in the future.
- Yes - The thought of curing Gaucher (Quite scary reading but sure further questions would help)

- No - I was aware of gene therapy in very vague terms before reading your paper. Now I have read it, I feel both very positive about it (in the sense that it offers the possibility for a wonderful new cure for a disease which was previous thought of as being incurable) and very negative (in the sense that the process itself sounds extremely traumatic for the patient. In fact it has provoked an emotional reaction in me which I was surprised to experience!). I feel it would be suitable for someone who was suffering very much from Gaucher Disease, perhaps a Type 3 patient or a newly diagnosed Type 1 patient, but not for someone like me whose disease is relatively controlled and who is a seasoned veteran of ERT and doesn't find it that bad. I should add that I have answered question 6 (fully licensed treatment – said no) below from that perspective but I would perhaps answer differently if I had been newly diagnosed and was faced with the prospect of a lifetime of ERT.

8 References

1. Gaucher P (1882) De l'épithélioma primitif de la rate. Hypertrophie idiopathique de la rate sans leucémie. Paris).
2. Bloem T, Groen J, & Postma C (1936) Gaucher's disease. *QJM* 5(4):517 - 523.
3. Brady RO, Kanfer JN, Bradley RM, & Shapiro D (1966) Demonstration of a deficiency of glucocerebrosidase in Gaucher's disease. *J Clin Invest* 45(7):1112-1115.
4. Ginns EI, *et al.* (1985) Gene mapping and leader polypeptide sequence of human glucocerebrosidase: implications for Gaucher disease. *Proc Natl Acad Sci U S A* 82(20):7101-7105.
5. Rath A & Marpillat N (2013) Prevalence of rare diseases: Bibliographic data, OrphaNet Report Series. in *Rare Diseases Collection*, ed Kremp O.
6. Horowitz M, *et al.* (1998) Prevalence of glucocerebrosidase mutations in the Israeli Ashkenazi Jewish population. *Hum Mutat* 12(4):240-244.
7. Fuller M, Meikle PJ, & Hopwood JJ (2006) Epidemiology of lysosomal storage disorders. *Fabry Disease: Perspectives from 5 years of FOS*, eds Mehta A, Beck M, & Sunder-Plassmann G (Oxford PharmaGenesis, Oxford).
8. Filocamo M & Morrone A (2011) Lysosomal storage disorders: molecular basis and laboratory testing. *Hum Genomics* 5(3):156-169.
9. Beck M (2001) Variable clinical presentation in lysosomal storage disorders. *J Inherit Metab Dis* 24 Suppl 2:47-45.
10. Bedilu R, *et al.* (2002) Variable clinical presentation of lysosomal beta-mannosidosis in patients with null mutations. *Mol Genet Metab* 77(4):282-290.
11. Brady RO, Kanfer JN, & Shapiro D (1965) Metabolism of glucocerebrosides II. Evidence of an enzymatic deficiency in Gaucher's disease. *Biochem Biophys Res Commun* 18:221-225.
12. Pentchev PG, Brady RO, Hibbert SR, Gal AE, & Shapiro D (1973) Isolation and characterization of glucocerebrosidase from human placental tissue. *J Biol Chem* 248(15):5256-5261.
13. Tsuji S, *et al.* (1987) A mutation in the human glucocerebrosidase gene in neuronopathic Gaucher's disease. *N Engl J Med* 316(10):570-575.
14. Tsuji S, *et al.* (1988) Genetic heterogeneity in type 1 Gaucher disease: multiple genotypes in Ashkenazic and non-Ashkenazic individuals. *Proc Natl Acad Sci U S A* 85(7):2349-2352.
15. De Duve C (1964) Principles of tissue fractionation. *J Theor Biol* 6(1):33-59.
16. Appelmans F, Wattiaux R, & Duve CD (1955) Tissue fractionation studies: Association of acid phosphatase with a special class of cytoplasmic granules in rat liver. *Biochemical Journal* 59(3):438-445.
17. Dunn WA, Jr. (1990) Studies on the mechanisms of autophagy: formation of the autophagic vacuole. *J Cell Biol* 110(6):1923-1933.
18. Dunn WA, Jr. (1990) Studies on the mechanisms of autophagy: maturation of the autophagic vacuole. *J Cell Biol* 110(6):1935-1945.
19. Bainton DF (1981) The discovery of lysosomes. *J Cell Biol* 91(3 Pt 2):66s-76s.
20. Natowicz MR, Chi MM, Lowry OH, & Sly WS (1979) Enzymatic identification of mannose 6-phosphate on the recognition marker for receptor-mediated pinocytosis of beta-glucuronidase by human fibroblasts. *Proc Natl Acad Sci U S A* 76(9):4322-4326.
21. Brown WJ, Goodhouse J, & Farquhar MG (1986) Mannose-6-phosphate receptors for lysosomal enzymes cycle between the Golgi complex and endosomes. *J Cell Biol* 103(4):1235-1247.

22. Wenger DA, Sattler M, Clark C, & Wharton C (1976) I-cell disease: activities of lysosomal enzymes toward natural and synthetic substrates. *Life Sci* 19(3):413-420.
23. Reczek D, *et al.* (2007) LIMP-2 is a receptor for lysosomal mannose-6-phosphate-independent targeting of beta-glucocerebrosidase. *Cell* 131(4):770-783.
24. Rijnboutt S, Aerts HM, Geuze HJ, Tager JM, & Strous GJ (1991) Mannose 6-phosphate-independent membrane association of cathepsin D, glucocerebrosidase, and sphingolipid-activating protein in HepG2 cells. *J Biol Chem* 266(8):4862-4868.
25. Jovic M, *et al.* (2012) Two phosphatidylinositol 4-kinases control lysosomal delivery of the Gaucher disease enzyme, beta-glucocerebrosidase. *Mol Biol Cell* 23(8):1533-1545.
26. Devine EA, Smith M, Arredondo-Vega FX, Shafit-Zagardo B, & Desnick RJ (1982) Chromosomal localization of the gene for Gaucher disease. *Prog Clin Biol Res* 95:511-534.
27. Horowitz M, *et al.* (1989) The human glucocerebrosidase gene and pseudogene: structure and evolution. *Genomics* 4(1):87-96.
28. Sorge J, Kuhl W, West C, & Beutler E (1987) Complete correction of the enzymatic defect of type I Gaucher disease fibroblasts by retroviral-mediated gene transfer. *Proc Natl Acad Sci U.S.A.* 84(4):906-909.
29. Svobodova E, *et al.* (2011) Glucocerebrosidase gene has an alternative upstream promoter, which has features and expression characteristic of housekeeping genes. *Blood Cells Mol Dis* 46(3):239-245.
30. Dvir H, *et al.* (2003) X-ray structure of human acid-beta-glucosidase, the defective enzyme in Gaucher disease. *EMBO Rep* 4(7):704-709.
31. Hruska KS, LaMarca ME, Scott CR, & Sidransky E (2008) Gaucher disease: mutation and polymorphism spectrum in the glucocerebrosidase gene (GBA). *Hum Mutat* 29(5):567-583.
32. Iselius L, Hillborg PO, & Lindsten J (1989) The distribution of the gene for the juvenile type of Gaucher disease in Sweden. *Acta Paediatr Scand* 78(4):592-596.
33. Charrow J, *et al.* (2000) The Gaucher registry: demographics and disease characteristics of 1698 patients with Gaucher disease. *Arch Intern Med* 160(18):2835-2843.
34. Liou B, *et al.* (2006) Analyses of variant acid beta-glucosidases: effects of Gaucher disease mutations. *J Biol Chem* 281(7):4242-4253.
35. Offman MN, Krol M, Silman I, Sussman JL, & Futerman AH (2010) Molecular basis of reduced glucosylceramidase activity in the most common Gaucher disease mutant, N370S. *J Biol Chem* 285(53):42105-42114.
36. Wei RR, *et al.* (2011) X-ray and biochemical analysis of N370S mutant human acid beta-glucosidase. *J Biol Chem* 286(1):299-308.
37. Grace ME, Newman KM, Scheinker V, Berg-Fussman A, & Grabowski GA (1994) Analysis of human acid beta-glucosidase by site-directed mutagenesis and heterologous expression. *J Biol Chem* 269(3):2283-2291.
38. Beutler E, Gelbart T, Kuhl W, Sorge J, & West C (1991) Identification of the second common Jewish Gaucher disease mutation makes possible population-based screening for the heterozygous state. *Proc Natl Acad Sci U S A* 88(23):10544-10547.
39. Orenstein M, Barbouth D, Bodamer OA, & Weinreb NJ (2014) Patients with type 1 Gaucher disease in South Florida, USA: demographics, genotypes, disease severity and treatment outcomes. *Orphanet Journal of Rare Diseases* 9(1):45.

40. Biegstraaten M, *et al.* (2011) A monozygotic twin pair with highly discordant Gaucher phenotypes. *Blood Cells Mol Dis* 46(1):39-41.
41. Lachmann RH, Grant IR, Halsall D, & Cox TM (2004) Twin pairs showing discordance of phenotype in adult Gaucher's disease. *QJM* 97(4):199-204.
42. Sibille A, Eng CM, Kim SJ, Pastores G, & Grabowski GA (1993) Phenotype/genotype correlations in Gaucher disease type I: clinical and therapeutic implications. *Am J Hum Genet* 52(6):1094-1101.
43. Fairley C, *et al.* (2008) Phenotypic heterogeneity of N370S homozygotes with type I Gaucher disease: an analysis of 798 patients from the ICGG Gaucher Registry. *J Inherit Metab Dis* 31(6):738-744.
44. Goker-Alpan O, *et al.* (2005) Divergent phenotypes in Gaucher disease implicate the role of modifiers. *J Med Genet* 42(6):e37.
45. Velayati A, *et al.* (2011) A mutation in SCARB2 is a modifier in Gaucher disease. *Hum Mutat* 32(11):1232-1238.
46. Zhang CK, *et al.* (2012) Genome-wide association study of N370S homozygous Gaucher disease reveals the candidacy of CLN8 gene as a genetic modifier contributing to extreme phenotypic variation. *Am J Hematol* 87(4):377-383.
47. Tylki-Szymanska A, *et al.* (2007) Non-neuronopathic Gaucher disease due to saposin C deficiency. *Clin Genet* 72(6):538-542.
48. Haddad SE, *et al.* (2012) CLN5 and CLN8 protein association with ceramide synthase: biochemical and proteomic approaches. *Electrophoresis* 33(24):3798-3809.
49. Thomas AS, Mehta A, & Hughes DA (2014) Gaucher disease: haematological presentations and complications. *Br J Haematol* 165(4):427-440.
50. Mikosch P & Hughes D (2010) An overview on bone manifestations in Gaucher disease. *Wien Med Wochenschr* 160(23-24):609-624.
51. Mignot C, Doummar D, Maire I, & De Villemeur TB (2006) Type 2 Gaucher disease: 15 new cases and review of the literature. *Brain Dev* 28(1):39-48.
52. Chen M & Wang J (2008) Gaucher disease: review of the literature. *Arch Path Lab Med* 132(5):851-853.
53. Cox TM (2010) Gaucher disease: clinical profile and therapeutic developments. *Biologics* 4:299-313.
54. Schueler UH, *et al.* (2004) Correlation between enzyme activity and substrate storage in a cell culture model system for Gaucher disease. *J Inherit Metab Dis* 27(5):649-658.
55. Cassinerio E, Graziadei G, & Poggiali E (2014) Gaucher disease: a diagnostic challenge for internists. *Eur J Intern Med* 25(2):117-124.
56. Bembi B, *et al.* (2003) Gaucher's disease with Parkinson's disease: clinical and pathological aspects. *Neurology* 61(1):99-101.
57. Tayebi N, *et al.* (2001) Gaucher disease and parkinsonism: a phenotypic and genotypic characterization. *Mol Genet Metab* 73(4):313-321.
58. Davidson C (1942) Disturbance in lipid metabolism and the central nervous system. *Mount Sinai Journal of Medicine* 9:389-406.
59. Neudorfer O, *et al.* (1996) Occurrence of Parkinson's syndrome in type I Gaucher disease. *QJM* 89(9):691-694.
60. Tayebi N, *et al.* (2003) Gaucher disease with parkinsonian manifestations: does glucocerebrosidase deficiency contribute to a vulnerability to parkinsonism? *Mol Genet Metab* 79(2):104-109.
61. Lwin A, Orvisky E, Goker-Alpan O, LaMarca ME, & Sidransky E (2004) Glucocerebrosidase mutations in subjects with parkinsonism. *Mol Genet Metab* 81(1):70-73.

62. Osellame LD, *et al.* (2013) Mitochondria and quality control defects in a mouse model of Gaucher disease - links to Parkinson's disease. *Cell Metab* 17(6):941-953.
63. Ron I, Rapaport D, & Horowitz M (2010) Interaction between parkin and mutant glucocerebrosidase variants: a possible link between Parkinson disease and Gaucher disease. *Hum Mol Genet* 19(19):3771-3781.
64. Dermentzaki G, Dimitriou E, Xilouri M, Michelakakis H, & Stefanis L (2013) Loss of beta-glucocerebrosidase activity does not affect alpha-synuclein levels or lysosomal function in neuronal cells. *PLoS One* 8(4):e60674.
65. Patterson MC, *et al.* (1993) Isolated horizontal supranuclear gaze palsy as a marker of severe systemic involvement in Gaucher's disease. *Neurology* 43(10):1993-1997.
66. Wong K, *et al.* (2004) Neuropathology provides clues to the pathophysiology of Gaucher disease. *Mol Genet Metab* 82(3):192-207.
67. Vitner EB, Farfel-Becker T, Eilam R, Biton I, & Futerman AH (2012) Contribution of brain inflammation to neuronal cell death in neuronopathic forms of Gaucher's disease. *Brain* 135(Pt 6):1724-1735.
68. Farfel-Becker T, *et al.* (2014) Neuronal accumulation of glucosylceramide in a mouse model of neuronopathic Gaucher disease leads to neurodegeneration. *Hum Mol Genet* 23(4):843-854.
69. Sun Y & Grabowski GA (2010) Impaired autophagosomes and lysosomes in neuronopathic Gaucher disease. *Autophagy* 6(5):648-649.
70. Mignot C, *et al.* (2003) Perinatal-lethal Gaucher disease. *Am J Med Genet A* 120a(3):338-344.
71. Svennerholm L, Erikson A, Groth CG, Ringden O, & Mansson JE (1991) Norrbottnian type of Gaucher disease - clinical, biochemical and molecular biology aspects: successful treatment with bone marrow transplantation. *Dev Neurosci* 13(4-5):345-351.
72. Pasmanik-Chor M, *et al.* (1996) The glucocerebrosidase D409H mutation in Gaucher disease. *Biochem Mol Med* 59(2):125-133.
73. Bohlega S, Kambouris M, Shahid M, Al Homsy M, & Al Sous W (2000) Gaucher disease with oculomotor apraxia and cardiovascular calcification (Gaucher type IIIC). *Neurology* 54(1):261-263.
74. Weinreb NJ, *et al.* (2008) Life expectancy in Gaucher disease type 1. *Am J Hematol* 83(12):896-900.
75. Abrahamov A, *et al.* (1995) Gaucher's disease variant characterised by progressive calcification of heart valves and unique genotype. *Lancet* 346(8981):1000-1003.
76. A. C, *et al.* (1995) Unusual expression of Gaucher's disease: cardiovascular calcifications in three sibs homozygous for the D409H mutation. *Journal of Medical Genetics* 32(9):740-742.
77. Germain DP (2004) Gaucher's disease: a paradigm for interventional genetics. *Clin Genet* 65(2):77-86.
78. Faden MA, Krakow D, Ezgu F, Rimoin DL, & Lachman RS (2009) The Erlenmeyer flask bone deformity in the skeletal dysplasias. *Am J Med Genet A* 149A(6):1334-1345.
79. Lutsky KF & Tejwani NC (2007) Orthopaedic manifestations of Gaucher disease. *Bull NYU Hosp Jt Dis* 65(1):37-42.
80. Mucci JM, *et al.* (2013) Uncoupling of osteoblast-osteoclast regulation in a chemical murine model of Gaucher disease. *Gene* 532(2):186-191.

81. Franz-Odenaal TA, Hall BK, & Witten PE (2006) Buried alive: how osteoblasts become osteocytes. *Dev Dyn* 235(1):176-190.
82. Fuller K, Thong JT, Breton BC, & Chambers TJ (1994) Automated three-dimensional characterization of osteoclastic resorption lacunae by stereoscopic scanning electron microscopy. *J Bone Miner Res* 9(1):17-23.
83. Gori F, *et al.* (2000) The expression of osteoprotegerin and RANK ligand and the support of osteoclast formation by stromal-osteoblast lineage cells is developmentally regulated. *Endocrinology* 141(12):4768-4776.
84. Parfitt AM (1994) Osteonal and hemi-osteonal remodeling: the spatial and temporal framework for signal traffic in adult human bone. *J Cell Biochem* 55(3):273-286.
85. Hollak CE, Evers L, Aerts JM, & van Oers MH (1997) Elevated levels of M-CSF, sCD14 and IL8 in type 1 Gaucher disease. *Blood Cells Mol Dis* 23(2):201-212.
86. Reed M, Baker RJ, Mehta AB, & Hughes DA (2013) Enhanced differentiation of osteoclasts from mononuclear precursors in patients with Gaucher disease. *Blood Cells Mol Dis* 51(3):185-194.
87. Mucci JM, *et al.* (2012) Induction of osteoclastogenesis in an in vitro model of Gaucher disease is mediated by T cells via TNF-alpha. *Gene* 509(1):51-59.
88. Lamghari M, Barrias CC, Sa Miranda C, & Barbosa MA (2005) Recombinant glucocerebrosidase uptake by Gaucher disease human osteoblast culture model. *Blood Cells Mol Dis* 35(3):348-354.
89. Mistry PK, *et al.* (2010) Glucocerebrosidase gene-deficient mouse recapitulates Gaucher disease displaying cellular and molecular dysregulation beyond the macrophage. *Proc Natl Acad Sci U S A* 107(45):19473-19478.
90. Barak V, *et al.* (1999) Cytokines in Gaucher's disease. *Eur Cytokine Netw* 10(2):205-210.
91. Allen MJ, Myer BJ, Khokher AM, Rushton N, & Cox TM (1997) Pro-inflammatory cytokines and the pathogenesis of Gaucher's disease: increased release of interleukin-6 and interleukin-10. *Qjm* 90(1):19-25.
92. Bendre MS, *et al.* (2003) Interleukin-8 stimulation of osteoclastogenesis and bone resorption is a mechanism for the increased osteolysis of metastatic bone disease. *Bone* 33(1):28-37.
93. Campeau PM, *et al.* (2009) Characterization of Gaucher disease bone marrow mesenchymal stromal cells reveals an altered inflammatory secretome. *Blood* 114(15):3181-3190.
94. Michelakakis H, *et al.* (1996) Plasma tumor necrosis factor- α (TNF- α) levels in Gaucher disease. *Biochim Biophys Acta* 1317(3):219-222.
95. Salky B, Kreel I, Gelernt I, Bauer J, & Aufses AH, Jr. (1979) Splenectomy for Gaucher's disease. *Ann Surg* 190(5):592-594.
96. Jmoudiak M & Futerman AH (2005) Gaucher disease: pathological mechanisms and modern management. *Br J Haematol* 129(2):178-188.
97. Cox TM, *et al.* (2008) Management of non-neuronopathic Gaucher disease with special reference to pregnancy, splenectomy, bisphosphonate therapy, use of biomarkers and bone disease monitoring. *J Inherit Metab Dis* 31(3):319-336.
98. Hoogerbrugge PM, *et al.* (1995) Allogeneic bone marrow transplantation for lysosomal storage diseases. The European Group for Bone Marrow Transplantation. *Lancet* 345(8962):1398-1402.
99. Hobbs JR, Jones KH, Shaw PJ, Lindsay I, & Hancock M (1987) Beneficial effect of pre-transplant splenectomy on displacement bone marrow transplantation for Gaucher's syndrome. *Lancet* 1(8542):1111-1115.

100. Ringden O, *et al.* (1995) Ten years' experience of bone marrow transplantation for Gaucher disease. *Transplantation* 59(6):864-870.
101. Erikson A, *et al.* (1990) Clinical and biochemical outcome of marrow transplantation for Gaucher disease of the Norrbottnian type. *Acta Paediatr Scand* 79(6-7):680-685.
102. Sawkar AR, D'Haeze W, & Kelly JW (2006) Therapeutic strategies to ameliorate lysosomal storage disorders--a focus on Gaucher disease. *Cell Mol Life Sci* 63(10):1179-1192.
103. Flomenberg N, *et al.* (2004) Impact of HLA class I and class II high-resolution matching on outcomes of unrelated donor bone marrow transplantation: HLA-C mismatching is associated with a strong adverse effect on transplantation outcome. *Blood* 104(7):1923-1930.
104. Giebel S, *et al.* (2003) Low incidence of severe acute graft-versus-host disease in children given haematopoietic stem cell transplantation from unrelated donors prospectively matched for HLA class I and II alleles with high-resolution molecular typing. *Bone Marrow Transplant* 31(11):987-993.
105. Giralt S, *et al.* (2014) Optimizing autologous stem cell mobilization strategies to improve patient outcomes: consensus guidelines and recommendations. *Biol Blood Marrow Transplant* 20(3):295-308.
106. Ito S & Barrett AJ (2013) Gauchers disease-a reappraisal of hematopoietic stem cell transplantation. *Pediatr Hematol Oncol* 30(2):61-70.
107. Somaraju UR & Tadepalli K (2012) Hematopoietic stem cell transplantation for Gaucher disease. *Cochrane Database Syst Rev* 7:Cd006974.
108. Desnick RJ & Schuchman EH (2012) Enzyme replacement therapy for lysosomal diseases: lessons from 20 years of experience and remaining challenges. *Annu Rev Genomics Hum Genet* 13:307-335.
109. Barton NW, *et al.* (1991) Replacement therapy for inherited enzyme deficiency--macrophage-targeted glucocerebrosidase for Gaucher's disease. *N Engl J Med* 324(21):1464-1470.
110. Dale GL & Beutler E (1976) Enzyme replacement therapy in Gaucher's disease: a rapid, high-yield method for purification of glucocerebrosidase. *Proc Natl Acad Sci U S A* 73(12):4672-4674.
111. Furbish FS, Blair HE, Shiloach J, Pentchev PG, & Brady RO (1977) Enzyme replacement therapy in Gaucher's disease: large-scale purification of glucocerebrosidase suitable for human administration. *Proc Natl Acad Sci U S A* 74(8):3560-3563.
112. Brady RO, Pentchev PG, Gal AE, Hibbert SR, & Dekaban AS (1974) Replacement therapy for inherited enzyme deficiency. Use of purified glucocerebrosidase in Gaucher's disease. *N Engl J Med* 291(19):989-993.
113. Brady RO, Murray GJ, & Barton NW (1994) Modifying exogenous glucocerebrosidase for effective replacement therapy in Gaucher disease. *J Inherit Metab Dis* 17(4):510-519.
114. Beutler E, Dale GL, Guinto DE, & Kuhl W (1977) Enzyme replacement therapy in Gaucher's disease: preliminary clinical trial of a new enzyme preparation. *Proc Natl Acad Sci U S A* 74(10):4620-4623.
115. Belchetz PE, Crawley JC, Braidman IP, & Gregoriadis G (1977) Treatment of Gaucher's disease with liposome-entrapped glucocerebrosidase: beta-glucosidase. *Lancet* 2(8029):116-117.
116. Furbish FS, Steer CJ, Barranger JA, Jones EA, & Brady RO (1978) The uptake of native and desialylated glucocerebrosidase by rat hepatocytes and Kupffer cells. *Biochem Biophys Res Commun* 81(3):1047-1053.

117. Thorpe SR, Fiddler MB, & Desnick RJ (1974) Enzyme therapy IV. A method for determining the in vivo fate of bovine beta-glucuronidase in beta-glucuronidase deficient mice. *Biochem Biophys Res Commun* 61(4):1464-1470.
118. Johnson WG, *et al.* (1973) Intravenous injection of purified hexosaminidase A into a patient with Tay-Sachs disease. *Birth Defects Orig Artic Ser* 9(2):120-124.
119. Stahl PD, Rodman JS, Miller MJ, & Schlesinger PH (1978) Evidence for receptor-mediated binding of glycoproteins, glycoconjugates, and lysosomal glycosidases by alveolar macrophages. *Proc Natl Acad Sci U S A* 75(3):1399-1403.
120. Takasaki S, *et al.* (1984) Structure of the N-asparagine-linked oligosaccharide units of human placental beta-glucocerebrosidase. *J Biol Chem* 259(16):10112-10117.
121. Furbish FS, Steer CJ, Krett NL, & Barranger JA (1981) Uptake and distribution of placental glucocerebrosidase in rat hepatic cells and effects of sequential deglycosylation. *Biochim Biophys Acta* 673(4):425-434.
122. Goldman DP, Clarke AE, & Garber AM (1992) Creating the costliest orphan. The Orphan Drug Act in the development of Ceredase. *Int J Technol Assess Health Care* 8(4):583-597.
123. Grabowski GA, *et al.* (1995) Enzyme therapy in type 1 Gaucher disease: comparative efficacy of mannose-terminated glucocerebrosidase from natural and recombinant sources. *Ann Intern Med* 122(1):33-39.
124. Zimran A, *et al.* (1995) Replacement therapy with imiglucerase for type 1 Gaucher's disease. *Lancet* 345(8963):1479-1480.
125. Zimran A, *et al.* (2010) Phase 1/2 and extension study of velaglucerase alfa replacement therapy in adults with type 1 Gaucher disease: 48-month experience. *Blood* 115(23):4651-4656.
126. Aerts JM, Yasothan U, & Kirkpatrick P (2010) Velaglucerase alfa. *Nat Rev Drug Discov* 9(11):837-838.
127. Zimran A, *et al.* (2011) Pivotal trial with plant cell-expressed recombinant glucocerebrosidase, taliglucerase alfa, a novel enzyme replacement therapy for Gaucher disease. *Blood* 118(22):5767-5773.
128. Zimran A, Loveday K, Fratazzi C, & Elstein D (2007) A pharmacokinetic analysis of a novel enzyme replacement therapy with Gene-Activated human glucocerebrosidase (GA-GCB) in patients with type 1 Gaucher disease. *Blood Cells Mol Dis* 39(1):115-118.
129. Elbein AD, Tropea JE, Mitchell M, & Kaushal GP (1990) Kifunensine, a potent inhibitor of the glycoprotein processing mannosidase I. *J Biol Chem* 265(26):15599-15605.
130. Brumshtein B, *et al.* (2010) Characterization of gene-activated human acid-beta-glucosidase: crystal structure, glycan composition, and internalization into macrophages. *Glycobiology* 20(1):24-32.
131. Hollak CE (2012) An evidence-based review of the potential benefits of taliglucerase alfa in the treatment of patients with Gaucher disease. *Core Evid* 7:15-20.
132. Traynor K (2012) Taliglucerase alfa approved for Gaucher disease. *Am J Health Syst Pharm* 69(12):1009.
133. Shaaltiel Y, *et al.* (2007) Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system. *Plant Biotechnol J* 5(5):579-590.
134. Ben Turkia H, *et al.* (2013) Velaglucerase alfa enzyme replacement therapy compared with imiglucerase in patients with Gaucher disease. *Am J Hematol* 88(3):179-184.

135. van Dussen L, *et al.* (2012) Effects of switching from a reduced dose imiglucerase to velaglucerase in type 1 Gaucher disease: clinical and biochemical outcomes. *Haematologica* 97(12):1850-1854.
136. Serratrice C, *et al.* (2014) Effects of switching from imiglucerase to velaglucerase alfa without dose reduction nor wash out in type 1 Gaucher disease. *Blood Cells Mol Dis*.
137. Elstein D, *et al.* (2012) Booster-effect with velaglucerase alfa in patients with Gaucher disease switched from long-term imiglucerase therapy: Early Access Program results from Jerusalem. *Blood Cells Mol. Dis.* 48(1):45–50.
138. van Dussen L, *et al.* (2013) Taliglucerase alfa leads to favorable bone marrow responses in patients with type I Gaucher disease. *Blood Cells Mol Dis* 50(3):206-211.
139. Pastores GM, *et al.* (2014) A Phase 3, multicenter, open-label, switchover trial to assess the safety and efficacy of taliglucerase alfa, a plant cell-expressed recombinant human glucocerebrosidase, in adult and pediatric patients with Gaucher disease previously treated with imiglucerase. *Blood Cells Mol Dis*.
140. Pfizer (2014) Pfizer and Protalix BioTherapeutics Announce FDA Approval of Pediatric Indication for ELELYSO™ (taliglucerase alfa) for Injection, for Intravenous Use for the Treatment of Type 1 Gaucher Disease.
141. FDA (2014) Press Announcements - FDA approves new orphan drug to treat a form of Gaucher disease.
142. Hollak CE, *et al.* (2010) Force majeure: therapeutic measures in response to restricted supply of imiglucerase (Cerezyme) for patients with Gaucher disease. *Blood Cells Mol Dis* 44(1):41-47.
143. Cox TM (2010) Recommendations for treating patients with Gaucher disease with emerging enzyme products. *Blood Cells Mol Dis* 44(2):84-85.
144. van Dussen L, Biegstraaten M, Hollak CE, & Dijkgraaf MG (2014) Cost-effectiveness of enzyme replacement therapy for type 1 Gaucher disease. *Orphanet J Rare Dis* 9(1).
145. Connock M, *et al.* (2006) The clinical effectiveness and cost-effectiveness of enzyme replacement therapy for Gaucher's disease: a systematic review. *Health Technol Assess* 10(24):iii-iv, ix-136.
146. Drelichman G, *et al.* (2007) Clinical consequences of interrupting enzyme replacement therapy in children with type 1 Gaucher disease. *J Pediatr* 151(2):197-201.
147. Zimran A, Altarescu G, & Elstein D (2011) Nonprecipitous changes upon withdrawal from imiglucerase for Gaucher disease because of a shortage in supply. *Blood Cells Mol Dis* 46(1):111-114.
148. Nagral A, *et al.* (2011) Recombinant macrophage targeted enzyme replacement therapy for Gaucher disease in India. *Indian Pediatr* 48(10):779-784.
149. Shah U, Nadeem N, Husen Y, & Fadoo Z (2007) Imiglucerase treatment in Gaucher's disease. *J Ayub Med Coll Abbottabad* 19(2):56-59.
150. Chan LL & Lin HP (2002) Enzyme replacement therapy for Gaucher Disease: the only experience in Malaysia. *Med J Malaysia* 57(3):348-352.
151. Beutler E, *et al.* (1995) The clinical course of treated and untreated Gaucher disease. A study of 45 patients. *Blood Cells Mol Dis* 21(2):86-108.
152. Charrow J, Dulisse B, Grabowski GA, & Weinreb NJ (2007) The effect of enzyme replacement therapy on bone crisis and bone pain in patients with type 1 Gaucher disease. *Clinical Genetics* 71(3):205-211.
153. Deegan PB, *et al.* (2011) Osseous manifestations of adult Gaucher disease in the era of enzyme replacement therapy. *Medicine (Baltimore)* 90(1):52-60.

154. Heaney RP, *et al.* (2000) Peak bone mass. *Osteoporos Int* 11(12):985-1009.
155. Mistry PK, *et al.* (2011) Osteopenia in Gaucher disease develops early in life: response to imiglucerase enzyme therapy in children, adolescents and adults. *Blood Cells Mol Dis* 46(1):66-72.
156. Andersson H, Kaplan P, Kacena K, & Yee J (2008) Eight-year clinical outcomes of long-term enzyme replacement therapy for 884 children with Gaucher disease type 1. *Pediatrics* 122(6):1182-1190.
157. Rosenthal DI, *et al.* (1995) Enzyme replacement therapy for Gaucher disease: skeletal responses to macrophage-targeted glucocerebrosidase. *Pediatrics* 96(4 Pt 1):629-637.
158. Grabowski GA, Leslie N, & Wenstrup R (1998) Enzyme therapy for Gaucher disease: the first 5 years. *Blood Rev* 12(2):115-133.
159. Ichikawa S & Hirabayashi Y (1998) Glucosylceramide synthase and glycosphingolipid synthesis. *Trends Cell Biol* 8(5):198-202.
160. Fischer PB, *et al.* (1995) The alpha-glucosidase inhibitor N-butyldeoxynojirimycin inhibits human immunodeficiency virus entry at the level of post-CD4 binding. *J Virol* 69(9):5791-5797.
161. Fischer PB, Karlsson GB, Butters TD, Dwek RA, & Platt FM (1996) N-butyldeoxynojirimycin-mediated inhibition of human immunodeficiency virus entry correlates with changes in antibody recognition of the V1/V2 region of gp120. *J Virol* 70(10):7143-7152.
162. Ficicioglu C (2008) Review of miglustat for clinical management in Gaucher disease type 1. *Ther Clin Risk Manag* 4(2):425-431.
163. Pastores GM, Elstein D, Hrebicek M, & Zimran A (2007) Effect of miglustat on bone disease in adults with type 1 Gaucher disease: a pooled analysis of three multinational, open-label studies. *Clin Ther* 29(8):1645-1654.
164. Belmatoug N, *et al.* (2011) Gastrointestinal disturbances and their management in miglustat-treated patients. *J Inherit Metab Dis* 34(5):991-1001.
165. Ko YM, Yamanaka T, Umeda M, & Suzuki Y (1983) Effects of thiol protease inhibitors on intracellular degradation of exogenous beta-galactosidase in cultured human skin fibroblasts. *Exp Cell Res* 148(2):525-529.
166. Suzuki Y, Sakuraba H, Hayashi K, Suzuki K, & Imahori K (1981) Beta-galactosidase-neuraminidase deficiency: restoration of beta-galactosidase activity by protease inhibitors. *J Biochem* 90(1):271-273.
167. Fan JQ, Ishii S, Asano N, & Suzuki Y (1999) Accelerated transport and maturation of lysosomal alpha-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. *Nat Med* 5(1):112-115.
168. Okumiya T, *et al.* (2007) Chemical chaperones improve transport and enhance stability of mutant alpha-glucosidases in glycogen storage disease type II. *Mol Genet Metab* 90(1):49-57.
169. Parenti G, *et al.* (2007) Pharmacological enhancement of mutated alpha-glucosidase activity in fibroblasts from patients with Pompe disease. *Mol Ther* 15(3):508-514.
170. Maegawa GH, *et al.* (2007) Pyrimethamine as a potential pharmacological chaperone for late-onset forms of GM2 gangliosidosis. *J Biol Chem* 282(12):9150-9161.
171. Tropak MB, Reid SP, Guiral M, Withers SG, & Mahuran D (2004) Pharmacological enhancement of beta-hexosaminidase activity in fibroblasts from adult Tay-Sachs and Sandhoff Patients. *J Biol Chem* 279(14):13478-13487.
172. Tropak MB, *et al.* (2008) Identification of pharmacological chaperones for Gaucher disease and characterization of their effects on beta-glucocerebrosidase

- by hydrogen/deuterium exchange mass spectrometry. *Chembiochem* 9(16):2650-2662.
173. Steet RA, *et al.* (2006) The iminosugar isofagomine increases the activity of N370S mutant acid beta-glucosidase in Gaucher fibroblasts by several mechanisms. *Proc Natl Acad Sci U S A* 103(37):13813-13818.
 174. Kornhaber GJ, *et al.* (2008) Isofagomine induced stabilization of glucocerebrosidase. *Chembiochem* 9(16):2643-2649.
 175. Maegawa GH, *et al.* (2009) Identification and characterization of ambroxol as an enzyme enhancement agent for Gaucher disease. *J Biol Chem* 284(35):23502-23516.
 176. Zimran A, Altarescu G, & Elstein D (2013) Pilot study using ambroxol as a pharmacological chaperone in type 1 Gaucher disease. *Blood Cells Mol Dis* 50(2):134-137.
 177. Sun Y, *et al.* (2011) Isofagomine in vivo effects in a neuronopathic Gaucher disease mouse. *PLoS One* 6(4):e19037.
 178. Sun Y, *et al.* (2012) Ex vivo and in vivo effects of isofagomine on acid beta-glucosidase variants and substrate levels in Gaucher disease. *J Biol Chem* 287(6):4275-4287.
 179. Hollak CE, van Weely S, van Oers MH, & Aerts JM (1994) Marked elevation of plasma chitotriosidase activity. A novel hallmark of Gaucher disease. *J Clin Invest* 93(3):1288-1292.
 180. Boot RG, *et al.* (2005) Marked differences in tissue-specific expression of chitinases in mouse and man. *J Histochem Cytochem* 53(10):1283-1292.
 181. Renkema GH, *et al.* (1997) Synthesis, sorting, and processing into distinct isoforms of human macrophage chitotriosidase. *Eur J Biochem* 244(2):279-285.
 182. Tybulewicz VL, *et al.* (1992) Animal model of Gaucher's disease from targeted disruption of the mouse glucocerebrosidase gene. *Nature* 357(6377):407-410.
 183. Enquist IB, *et al.* (2006) Effective cell and gene therapy in a murine model of Gaucher disease. *PNAS* 103(37):13819-13824.
 184. Enquist IB, *et al.* (2007) Murine models of acute neuronopathic Gaucher disease. *PNAS* 104(44):17483-17488.
 185. Sinclair GB, *et al.* (2007) Generation of a conditional knockout of murine glucocerebrosidase: utility for the study of Gaucher disease. *Mol Genet Metab* 90(2):148-156.
 186. Mizukami H, *et al.* (2002) Systemic inflammation in glucocerebrosidase-deficient mice with minimal glucosylceramide storage. *J Clin Invest* 109(9):1215-1221.
 187. Xu YH, Quinn B, Witte D, & Grabowski GA (2003) Viable mouse models of acid beta-glucosidase deficiency: the defect in Gaucher disease. *Am J Pathol* 163(5):2093-2101.
 188. Griffith F (1928) The significance of Pneumococcal types. *J Hyg (Lond)* 27(2):113-159.
 189. Avery OT, Macleod CM, & McCarty M (1944) Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of transformation by a deoxyribonucleic acid fraction isolated from pneumococcus type III. *J Exp Med* 79(2):137-158.
 190. Hill M & Huppert J (1970) Fate of exogenous mouse DNA in chicken fibroblasts in vitro. Non-conservative preservation. *Biochim Biophys Acta* 213(1):26-35.
 191. Borenfreund E & Bendich A (1961) A study on the penetration of mammalian cells by deoxyribonucleic acids. *J Biophys Biochem Cytol* 9(1):81-91.
 192. Majumdar A & Bose SK (1968) DNA mediated genetic transformation of a human cancerous cell line cultured in vitro. *Br J Cancer* 22(3):603-613.

193. Rabotti GF (1963) Incorporation of DNA into a mouse tumor in vivo and in vitro. *Exp Cell Res* 31:562-565.
194. Westphal H & Dulbecco R (1968) Viral DNA in polyoma- and SV40-transformed cell lines. *Proc Natl Acad Sci U S A* 59(4):1158-1165.
195. Sambrook J, Westphal H, Srinivasan PR, & Dulbecco R (1968) The integrated state of viral DNA in SV40-transformed cells. *Proc Natl Acad Sci U S A* 60(4):1288-1295.
196. Friedmann T & Roblin R (1972) Gene therapy for human genetic disease? *Science* 175(4025):949-955.
197. Maniatis T, *et al.* (1978) The isolation of structural genes from libraries of eucaryotic DNA. *Cell* 15(2):687-701.
198. Mulligan RC, Howard BH, & Berg P (1979) Synthesis of rabbit beta-globin in cultured monkey kidney cells following infection with a SV40 beta-globin recombinant genome. *Nature* 277(5692):108-114.
199. Wei CM, Gibson M, Spear PG, & Scolnick EM (1981) Construction and isolation of a transmissible retrovirus containing the src gene of Harvey murine sarcoma virus and the thymidine kinase gene of herpes simplex virus type 1. *J Virol* 39(3):935-944.
200. Shimotohno K & Temin HM (1981) Formation of infectious progeny virus after insertion of herpes simplex thymidine kinase gene into DNA of an avian retrovirus. *Cell* 26(1 Pt 1):67-77.
201. Tabin CJ, Hoffmann JW, Goff SP, & Weinberg RA (1982) Adaptation of a retrovirus as a eucaryotic vector transmitting the herpes simplex virus thymidine kinase gene. *Mol Cell Biol* 2(4):426-436.
202. Mann R, Mulligan RC, & Baltimore D (1983) Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* 33(1):153-159.
203. Markowitz D, Goff S, & Bank A (1988) A safe packaging line for gene transfer: separating viral genes on two different plasmids. *J Virol* 62(4):1120-1124.
204. Cone RD & Mulligan RC (1984) High-efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host range. *Proc Natl Acad Sci U S A* 81(20):6349-6353.
205. Miller AD, Jolly DJ, Friedmann T, & Verma IM (1983) A transmissible retrovirus expressing human hypoxanthine phosphoribosyltransferase (HPRT): gene transfer into cells obtained from humans deficient in HPRT. *Proc Natl Acad Sci U S A* 80(15):4709-4713.
206. Willis RC, *et al.* (1984) Partial phenotypic correction of human Lesch-Nyhan (hypoxanthine-guanine phosphoribosyltransferase-deficient) lymphoblasts with a transmissible retroviral vector. *J Biol Chem* 259(12):7842-7849.
207. Kantoff PW, *et al.* (1986) Correction of adenosine deaminase deficiency in cultured human T and B cells by retrovirus-mediated gene transfer. *Proc Natl Acad Sci U S A* 83(17):6563-6567.
208. Ledley FD, Grenett HE, Bartos DP, & Woo SL (1987) Retroviral mediated transfer and expression of human alpha 1-antitrypsin in cultured cells. *Gene* 61(1):113-118.
209. Fink JK, Correll PH, Perry LK, Brady RO, & Karlsson S (1990) Correction of glucocerebrosidase deficiency after retroviral-mediated gene transfer into hematopoietic progenitor cells from patients with Gaucher disease. *PNAS* 87(6):2334-2338.
210. Back AL, Kwok WW, Adam M, Collins SJ, & Hickstein DD (1990) Retroviral-mediated gene transfer of the leukocyte integrin CD18 subunit. *Biochem Biophys Res Commun* 171(2):787-795.

211. Huttner KM, Scangos GA, & Ruddle FH (1979) DNA-mediated gene transfer of a circular plasmid into murine cells. *Proc Natl Acad Sci U S A* 76(11):5820-5824.
212. Wigler M, *et al.* (1980) Transformation of mammalian cells with an amplifiable dominant-acting gene. *Proc Natl Acad Sci U S A* 77(6):3567-3570.
213. Pavlakis GN, Hizuka N, Gorden P, Seeburg P, & Hamer DH (1981) Expression of two human growth hormone genes in monkey cells infected by simian virus 40 recombinants. *Proc Natl Acad Sci U S A* 78(12):7398-7402.
214. Cline MJ, *et al.* (1980) Gene transfer in intact animals. *Nature* 284(5755):422-425.
215. Joyner A, Keller G, Phillips RA, & Bernstein A (1983) Retrovirus transfer of a bacterial gene into mouse haematopoietic progenitor cells. *Nature* 305(5934):556-558.
216. Cline MJ (1985) Perspectives for gene therapy: inserting new genetic information into mammalian cells by physical techniques and viral vectors. *Pharmacol Ther* 29(1):69-92.
217. Mercola KE, Stang HD, Browne J, Salser W, & Cline MJ (1980) Insertion of a new gene of viral origin into bone marrow cells of mice. *Science* 208(4447):1033-1035.
218. Wade N (1980) UCLA gene therapy racked by friendly fire. *Science* 210(4469):509-511.
219. Beutler E (2001) The Cline affair. *Mol Ther* 4(5):396-397.
220. Blaese RM, *et al.* (1995) T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. *Science* 270(5235):475-480.
221. Bordignon C, *et al.* (1995) Gene therapy in peripheral blood lymphocytes and bone marrow for ADA- immunodeficient patients. *Science* 270(5235):470-475.
222. Luens KM, *et al.* (1998) Thrombopoietin, kit ligand, and flk2/flt3 ligand together induce increased numbers of primitive hematopoietic progenitors from human CD34+Thy-1+Lin- cells with preserved ability to engraft SCID-hu bone. *Blood* 91(4):1206-1215.
223. Hanenberg H, *et al.* (1997) Optimization of fibronectin-assisted retroviral gene transfer into human CD34+ hematopoietic cells. *Hum Gene Ther* 8(18):2193-2206.
224. Aiuti A, *et al.* (2002) Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* 296(5577):2410-2413.
225. Montiel-Equihua CA, *et al.* (2012) The beta-globin locus control region in combination with the EF1alpha short promoter allows enhanced lentiviral vector-mediated erythroid gene expression with conserved multilineage activity. *Mol Ther* 20(7):1400-1409.
226. Gaspar HB, *et al.* (2011) Hematopoietic stem cell gene therapy for adenosine deaminase-deficient severe combined immunodeficiency leads to long-term immunological recovery and metabolic correction. *Sci Transl Med* 3(97):97ra80.
227. Candotti F, *et al.* (2012) Gene therapy for adenosine deaminase-deficient severe combined immune deficiency: clinical comparison of retroviral vectors and treatment plans. *Blood* 120(18):3635-3646.
228. Aiuti A, *et al.* (2009) Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *N Engl J Med* 360(5):447-458.
229. Mukherjee S & Thrasher AJ (2013) Gene therapy for PIDs: progress, pitfalls and prospects. *Gene* 525(2):174-181.
230. Puck JM, *et al.* (1993) The interleukin-2 receptor gamma chain maps to Xq13.1 and is mutated in X-linked severe combined immunodeficiency, SCIDX1. *Hum Mol Genet* 2(8):1099-1104.
231. Cavazzana-Calvo M, *et al.* (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* 288(5466):669-672.

232. Hacein-Bey-Abina S, *et al.* (2002) Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N Engl J Med* 346(16):1185-1193.
233. Gaspar HB, *et al.* (2004) Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet* 364(9452):2181-2187.
234. Hacein-Bey-Abina S, *et al.* (2008) Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest* 118(9):3132-3142.
235. Hacein-Bey-Abina S, *et al.* (2003) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 302(5644):415-419.
236. Howe SJ, *et al.* (2008) Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest* 118(9):3143-3150.
237. Ott MG, *et al.* (2006) Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat Med* 12(4):401-409.
238. Stein S, *et al.* (2010) Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. *Nat Med* 16(2):198-204.
239. Cavazzana-Calvo M, *et al.* (2010) Transfusion independence and HMGA2 activation after gene therapy of human β -thalassaemia. *Nature* 467(7313):318-322.
240. Raper SE, *et al.* (2003) Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab* 80(1-2):148-158.
241. Raper SE, *et al.* (2002) A pilot study of in vivo liver-directed gene transfer with an adenoviral vector in partial ornithine transcarbamylase deficiency. *Hum Gene Ther* 13(1):163-175.
242. Wilson JM (2009) Lessons learned from the gene therapy trial for ornithine transcarbamylase deficiency. *Mol Genet Metab* 96(4):151-157.
243. Yarborough M & Sharp RR (2009) Public trust and research a decade later: what have we learned since Jesse Gelsinger's death? *Mol Genet Metab* 97(1):4-5.
244. Bortin MM (1970) A compendium of reported human bone marrow transplants. *Transplantation* 9(6):571-587.
245. Choi JH, *et al.* (2014) Optimization of AAV expression cassettes to improve packaging capacity and transgene expression in neurons. *Mol Brain* 7:17.
246. Seto JT, Bengtsson NE, & Chamberlain JS (2014) Therapy of Genetic Disorders- Novel Therapies for Duchenne Muscular Dystrophy. *Curr Pediatr Rep* 2(2):102-112.
247. Boutin S, *et al.* (2010) Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. *Hum Gene Ther* 21(6):704-712.
248. Reeh M, *et al.* (2013) Presence of the coxsackievirus and adenovirus receptor (CAR) in human neoplasms: a multitumour array analysis. *Br J Cancer* 109(7):1848-1858.
249. Hareendran S, *et al.* (2013) Adeno-associated virus (AAV) vectors in gene therapy: immune challenges and strategies to circumvent them. *Rev Med Virol* 23(6):399-413.
250. Mingozzi F, *et al.* (2009) AAV-1-mediated gene transfer to skeletal muscle in humans results in dose-dependent activation of capsid-specific T cells. *Blood* 114(10):2077-2086.

251. Chen SJ, *et al.* (2013) Biodistribution of AAV8 vectors expressing human low-density lipoprotein receptor in a mouse model of homozygous familial hypercholesterolemia. *Hum Gene Ther Clin Dev* 24(4):154-160.
252. Palomeque J, *et al.* (2007) Efficiency of eight different AAV serotypes in transducing rat myocardium in vivo. *Gene Ther*, England, Vol 14, pp 989-997.
253. Keswani SG, *et al.* (2012) Pseudotyped adeno-associated viral vector tropism and transduction efficiencies in murine wound healing. *Wound Repair Regen* 20(4):592-600.
254. Foust KD, *et al.* (2009) Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nat Biotechnol* 27(1):59-65.
255. Rahim AA, *et al.* (2011) Intravenous administration of AAV2/9 to the fetal and neonatal mouse leads to differential targeting of CNS cell types and extensive transduction of the nervous system. *FASEB J* 25(10):3505-3518.
256. Nathwani AC, Davidoff AM, & Linch DC (2005) A review of gene therapy for haematological disorders. *Br J Haematol* 128(1):3-17.
257. Temin HM & Mizutani S (1970) RNA-dependent DNA polymerase in virions of Rous sarcoma virus. *Nature* 226(5252):1211-1213.
258. Baltimore D (1970) RNA-dependent DNA polymerase in virions of RNA tumour viruses. *Nature* 226(5252):1209-1211.
259. Bushman FD, Fujiwara T, & Craigie R (1990) Retroviral DNA integration directed by HIV integration protein in vitro. *Science* 249(4976):1555-1558.
260. Miyake K, Suzuki N, Matsuoka H, Tohyama T, & Shimada T (1998) Stable integration of human immunodeficiency virus-based retroviral vectors into the chromosomes of nondividing cells. *Hum Gene Ther* 9(4):467-475.
261. Schroder AR, *et al.* (2002) HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* 110(4):521-529.
262. Wu X, Li Y, Crise B, & Burgess SM (2003) Transcription start regions in the human genome are favored targets for MLV integration. *Science* 300(5626):1749-1751.
263. Mitchell RS, *et al.* (2004) Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biol* 2(8):E234.
264. Cattoglio C, *et al.* (2007) Hot spots of retroviral integration in human CD34+ hematopoietic cells. *Blood* 110(6):1770-1778.
265. Li Y, *et al.* (1992) Complete nucleotide sequence, genome organization, and biological properties of human immunodeficiency virus type 1 in vivo: evidence for limited defectiveness and complementation. *J Virol* 66(11):6587-6600.
266. Mancebo HS, *et al.* (1997) P-TEFb kinase is required for HIV Tat transcriptional activation in vivo and in vitro. *Genes Dev* 11(20):2633-2644.
267. Wei P, Garber ME, Fang SM, Fischer WH, & Jones KA (1998) A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell* 92(4):451-462.
268. Wilson W, *et al.* (1988) HIV expression strategies: ribosomal frameshifting is directed by a short sequence in both mammalian and yeast systems. *Cell* 55(6):1159-1169.
269. Jacks T, *et al.* (1988) Characterization of ribosomal frameshifting in HIV-1 gag-pol expression. *Nature* 331(6153):280-283.
270. Willey RL, Bonifacino JS, Potts BJ, Martin MA, & Klausner RD (1988) Biosynthesis, cleavage, and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160. *Proc Natl Acad Sci U S A* 85(24):9580-9584.
271. McCune JM, *et al.* (1988) Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus. *Cell* 53(1):55-67.

272. Lu M, Blacklow SC, & Kim PS (1995) A trimeric structural domain of the HIV-1 transmembrane glycoprotein. *Nat Struct Biol* 2(12):1075-1082.
273. Thali M, *et al.* (1993) Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. *J Virol* 67(7):3978-3988.
274. Sattentau QJ, Moore JP, Vignaux F, Traincard F, & Poignard P (1993) Conformational changes induced in the envelope glycoproteins of the human and simian immunodeficiency viruses by soluble receptor binding. *J Virol* 67(12):7383-7393.
275. Cronin J, Zhang XY, & Reiser J (2005) Altering the tropism of lentiviral vectors through pseudotyping. *Curr Gene Ther* 5(4):387-398.
276. Ratner L, *et al.* (1985) Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature* 313(6000):277-284.
277. Shehu-Xhilaga M, Crowe SM, & Mak J (2001) Maintenance of the Gag/Gag-Pol ratio is important for human immunodeficiency virus type 1 RNA dimerization and viral infectivity. *J Virol* 75(4):1834-1841.
278. Schatzl H, Gelderblom HR, Nitschko H, & von der Helm K (1991) Analysis of non-infectious HIV particles produced in presence of HIV proteinase inhibitor. *Arch Virol* 120(1-2):71-81.
279. Fu W, Gorelick RJ, & Rein A (1994) Characterization of human immunodeficiency virus type 1 dimeric RNA from wild-type and protease-defective virions. *J Virol* 68(8):5013-5018.
280. Kaplan AH, *et al.* (1993) Partial inhibition of the human immunodeficiency virus type 1 protease results in aberrant virus assembly and the formation of noninfectious particles. *J Virol* 67(7):4050-4055.
281. Navia MA, *et al.* (1989) Three-dimensional structure of aspartyl protease from human immunodeficiency virus HIV-1. *Nature* 337(6208):615-620.
282. Muriaux D, De Rocquigny H, Roques BP, & Paoletti J (1996) NCp7 activates HIV-1Lai RNA dimerization by converting a transient loop-loop complex into a stable dimer. *J Biol Chem* 271(52):33686-33692.
283. Mujeeb A, *et al.* (2007) Nucleocapsid protein-mediated maturation of dimer initiation complex of full-length SL1 stemloop of HIV-1: sequence effects and mechanism of RNA refolding. *Nucleic Acids Res* 35(6):2026-2034.
284. Feng YX, *et al.* (1996) HIV-1 nucleocapsid protein induces "maturation" of dimeric retroviral RNA in vitro. *Proc Natl Acad Sci U S A* 93(15):7577-7581.
285. Li S, Hill CP, Sundquist WI, & Finch JT (2000) Image reconstructions of helical assemblies of the HIV-1 CA protein. *Nature* 407(6802):409-413.
286. Briggs JA, Wilk T, Welker R, Krausslich HG, & Fuller SD (2003) Structural organization of authentic, mature HIV-1 virions and cores. *EMBO J* 22(7):1707-1715.
287. Lopez CS, *et al.* (2011) Determinants of the HIV-1 core assembly pathway. *Virology* 417(1):137-146.
288. Yuan X, Yu X, Lee TH, & Essex M (1993) Mutations in the N-terminal region of human immunodeficiency virus type 1 matrix protein block intracellular transport of the Gag precursor. *J Virol* 67(11):6387-6394.
289. Zhou W, Parent LJ, Wills JW, & Resh MD (1994) Identification of a membrane-binding domain within the amino-terminal region of human immunodeficiency virus type 1 Gag protein which interacts with acidic phospholipids. *J Virol* 68(4):2556-2569.
290. Temin HM (1964) Homology between RNA from Rous sarcoma virus and DNA from Rous sarcoma virus-infected cells. *Proc Natl Acad Sci U S A* 52:323-329.

291. di Marzo Veronese F, *et al.* (1986) Characterization of highly immunogenic p66/p51 as the reverse transcriptase of HTLV-III/LAV. *Science* 231(4743):1289-1291.
292. Telesnitsky A & Goff SP (1993) Two defective forms of reverse transcriptase can complement to restore retroviral infectivity. *EMBO J* 12(11):4433-4438.
293. Barat C, *et al.* (1989) HIV-1 reverse transcriptase specifically interacts with the anticodon domain of its cognate primer tRNA. *EMBO J* 8(11):3279-3285.
294. Sallafranque-Andreola ML, *et al.* (1989) Human immunodeficiency virus reverse transcriptase expressed in transformed yeast cells. Biochemical properties and interactions with bovine tRNA^{Lys}. *Eur J Biochem* 184(2):367-374.
295. Huber HE & Richardson CC (1990) Processing of the primer for plus strand DNA synthesis by human immunodeficiency virus 1 reverse transcriptase. *J Biol Chem* 265(18):10565-10573.
296. Wohrl BM & Moelling K (1990) Interaction of HIV-1 ribonuclease H with polypurine tract containing RNA-DNA hybrids. *Biochemistry* 29(44):10141-10147.
297. Panganiban AT & Fiore D (1988) Ordered interstrand and intrastrand DNA transfer during reverse transcription. *Science* 241(4869):1064-1069.
298. Butler SL, Hansen MS, & Bushman FD (2001) A quantitative assay for HIV DNA integration in vivo. *Nat Med* 7(5):631-634.
299. Devroe E, Engelman A, & Silver PA (2003) Intracellular transport of human immunodeficiency virus type 1 integrase. *J Cell Sci* 116(Pt 21):4401-4408.
300. Woerner AM & Marcus-Sekura CJ (1993) Characterization of a DNA binding domain in the C-terminus of HIV-1 integrase by deletion mutagenesis. *Nucleic Acids Res* 21(15):3507-3511.
301. Dolan J, Chen A, Weber IT, Harrison RW, & Leis J (2009) Defining the DNA substrate binding sites on HIV-1 integrase. *J Mol Biol* 385(2):568-579.
302. Kamata M, Nitahara-Kasahara Y, Miyamoto Y, Yoneda Y, & Aida Y (2005) Importin- α promotes passage through the nuclear pore complex of human immunodeficiency virus type 1 Vpr. *J Virol* 79(6):3557-3564.
303. Woodward CL, Prakobwanakit S, Mosessian S, & Chow SA (2009) Integrase interacts with nucleoporin NUP153 to mediate the nuclear import of human immunodeficiency virus type 1. *J Virol* 83(13):6522-6533.
304. Friedrich BM, *et al.* (2011) A functional role for ADAM10 in human immunodeficiency virus type-1 replication. *Retrovirology* 8:32.
305. Bushman FD & Craigie R (1991) Activities of human immunodeficiency virus (HIV) integration protein in vitro: specific cleavage and integration of HIV DNA. *Proc Natl Acad Sci U S A* 88(4):1339-1343.
306. Gaken JA, *et al.* (1996) Efficient retroviral infection of mammalian cells is blocked by inhibition of poly(ADP-ribose) polymerase activity. *J Virol* 70(6):3992-4000.
307. Van Maele B & Debyser Z (2005) HIV-1 integration: an interplay between HIV-1 integrase, cellular and viral proteins. *AIDS Rev* 7(1):26-43.
308. Cullen BR & Malim MH (1991) The HIV-1 Rev protein: prototype of a novel class of eukaryotic post-transcriptional regulators. *Trends Biochem Sci* 16(9):346-350.
309. Malim MH, Hauber J, Le SY, Maizel JV, & Cullen BR (1989) The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. *Nature* 338(6212):254-257.
310. Fischer U, Huber J, Boelens WC, Mattaj JW, & Luhrmann R (1995) The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell* 82(3):475-483.

311. Dingwall C, *et al.* (1989) Human immunodeficiency virus 1 tat protein binds trans-activation-responsive region (TAR) RNA in vitro. *Proc Natl Acad Sci U S A* 86(18):6925-6929.
312. Kao SY, Calman AF, Luciw PA, & Peterlin BM (1987) Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. *Nature* 330(6147):489-493.
313. Rayne F, Debaisieux S, Bonhoure A, & Beaumelle B (2010) HIV-1 Tat is unconventionally secreted through the plasma membrane. *Cell Biol Int* 34(4):409-413.
314. Frankel AD & Pabo CO (1988) Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* 55(6):1189-1193.
315. Lee KO, *et al.* (2005) Improved intracellular delivery of glucocerebrosidase mediated by the HIV-1 TAT protein transduction domain. *Biochem Biophys Res Commun* 337(2):701-707.
316. Flinterman M, *et al.* (2009) Delivery of therapeutic proteins as secretable TAT fusion products. *Mol Ther* 17(2):334-342.
317. Grubb JH, Vogler C, & Sly WS (2010) New strategies for enzyme replacement therapy for lysosomal storage diseases. *Rejuvenation Res* 13(2-3):229-236.
318. Zufferey R, Nagy D, Mandel RJ, Naldini L, & Trono D (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol* 15(9):871-875.
319. Kim VN, Mitrophanous K, Kingsman SM, & Kingsman AJ (1998) Minimal requirement for a lentivirus vector based on human immunodeficiency virus type 1. *J Virol* 72(1):811-816.
320. Ott DE (2002) Potential roles of cellular proteins in HIV-1. *Rev Med Virol* 12(6):359-374.
321. Skripkin E, Paillart JC, Marquet R, Ehresmann B, & Ehresmann C (1994) Identification of the primary site of the human immunodeficiency virus type 1 RNA dimerization in vitro. *Proc Natl Acad Sci U S A* 91(11):4945-4949.
322. Kutluay SB & Bieniasz PD (2010) Analysis of the initiating events in HIV-1 particle assembly and genome packaging. *PLoS Pathog* 6(11):e1001200.
323. Chukkapalli V, Oh SJ, & Ono A (2010) Opposing mechanisms involving RNA and lipids regulate HIV-1 Gag membrane binding through the highly basic region of the matrix domain. *Proc Natl Acad Sci U S A* 107(4):1600-1605.
324. Freed EO & Martin MA (1995) Virion incorporation of envelope glycoproteins with long but not short cytoplasmic tails is blocked by specific, single amino acid substitutions in the human immunodeficiency virus type 1 matrix. *J Virol* 69(3):1984-1989.
325. Murakami T & Freed EO (2000) Genetic evidence for an interaction between human immunodeficiency virus type 1 matrix and alpha-helix 2 of the gp41 cytoplasmic tail. *J Virol* 74(8):3548-3554.
326. Postler TS & Desrosiers RC (2013) The tale of the long tail: the cytoplasmic domain of HIV-1 gp41. *J Virol* 87(1):2-15.
327. Van Engelenburg SB, *et al.* (2014) Distribution of ESCRT machinery at HIV assembly sites reveals virus scaffolding of ESCRT subunits. *Science* 343(6171):653-656.
328. Meng B & Lever AM (2013) Wrapping up the bad news: HIV assembly and release. *Retrovirology* 10:5.
329. Ganser BK, Li S, Klishko VY, Finch JT, & Sundquist WI (1999) Assembly and analysis of conical models for the HIV-1 core. *Science* 283(5398):80-83.

330. Benjamin J, Ganser-Pornillos BK, Tivol WF, Sundquist WI, & Jensen GJ (2005) Three-dimensional structure of HIV-1 virus-like particles by electron cryotomography. *J Mol Biol* 346(2):577-588.
331. Cosson P (1996) Direct interaction between the envelope and matrix proteins of HIV-1. *EMBO J* 15(21):5783-5788.
332. Tedbury PR, Ablan SD, & Freed EO (2013) Global rescue of defects in HIV-1 envelope glycoprotein incorporation: implications for matrix structure. *PLoS Pathog* 9(11):e1003739.
333. Varmus HE (1982) Form and function of retroviral proviruses. *Science* 216(4548):812-820.
334. Yu SF, *et al.* (1986) Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. *Proc Natl Acad Sci U S A* 83(10):3194-3198.
335. Peng YF, *et al.* (2013) Alpha-fetoprotein promoter-driven Cre/LoxP-switched RNA interference for hepatocellular carcinoma tissue-specific target therapy. *PLoS One* 8(2):e53072.
336. Romero Z, *et al.* (2011) A tissue-specific, activation-inducible, lentiviral vector regulated by human CD40L proximal promoter sequences. *Gene Ther* 18(4):364-371.
337. Zhang F, *et al.* (2007) Lentiviral vectors containing an enhancer-less ubiquitously acting chromatin opening element (UCOE) provide highly reproducible and stable transgene expression in hematopoietic cells. *Blood* 110(5):1448-1457.
338. Zhang F, *et al.* (2010) A ubiquitous chromatin opening element (UCOE) confers resistance to DNA methylation-mediated silencing of lentiviral vectors. *Mol Ther* 18(9):1640-1649.
339. Brooks AR, *et al.* (2004) Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. *J Gene Med* 6(4):395-404.
340. Herbst F, *et al.* (2012) Extensive methylation of promoter sequences silences lentiviral transgene expression during stem cell differentiation in vivo. *Mol Ther* 20(5):1014-1021.
341. Vigna E & Naldini L (2000) Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy. *J Gene Med* 2(5):308-316.
342. Naldini L, *et al.* (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 272(5259):263-267.
343. Page KA, Landau NR, & Littman DR (1990) Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. *J Virol* 64(11):5270-5276.
344. Burns JC, Friedmann T, Driever W, Burrascano M, & Yee JK (1993) Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc Natl Acad Sci U S A* 90(17):8033-8037.
345. Dull T, *et al.* (1998) A third-generation lentivirus vector with a conditional packaging system. *J Virol* 72(11):8463-8471.
346. Wadia JS, Stan RV, & Dowdy SF (2004) Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med* 10(3):310-315.
347. Kaplan IM, Wadia JS, & Dowdy SF (2005) Cationic TAT peptide transduction domain enters cells by macropinocytosis. *J Control Release* 102(1):247-253.

348. Green M & Loewenstein PM (1988) Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. *Cell*, United States), Vol 55, pp 1179-1188.
349. Vives E, Brodin P, & Lebleu B (1997) A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J Biol Chem* 272(25):16010-16017.
350. Derossi D, Joliot AH, Chassaing G, & Prochiantz A (1994) The third helix of the Antennapedia homeodomain translocates through biological membranes. *J Biol Chem* 269(14):10444-10450.
351. Aints A, Guven H, Gahrton G, Smith CI, & Dilber MS (2001) Mapping of herpes simplex virus-1 VP22 functional domains for inter- and subcellular protein targeting. *Gene Ther* 8(14):1051-1056.
352. Mitchell DJ, Kim DT, Steinman L, Fathman CG, & Rothbard JB (2000) Polyarginine enters cells more efficiently than other polycationic homopolymers. *J Pept Res* 56(5):318-325.
353. Shen WC & Ryser HJ (1978) Conjugation of poly-L-lysine to albumin and horseradish peroxidase: a novel method of enhancing the cellular uptake of proteins. *Proc Natl Acad Sci U S A* 75(4):1872-1876.
354. Mi Z, Mai J, Lu X, & Robbins PD (2000) Characterization of a class of cationic peptides able to facilitate efficient protein transduction in vitro and in vivo. *Mol Ther*, United States), Vol 2, pp 339-347.
355. Tilstra J, *et al.* (2007) Protein transduction: identification, characterization and optimization. *Biochem Soc Trans*, England), Vol 35, pp 811-815.
356. Jones SW, *et al.* (2005) Characterisation of cell-penetrating peptide-mediated peptide delivery. *Br J Pharmacol* 145(8):1093-1102.
357. Schwarze SR, Ho A, Vocero-Akbani A, & Dowdy SF (1999) In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* 285(5433):1569-1572.
358. Zhang XY, Dinh A, Cronin J, Li SC, & Reiser J (2008) Cellular uptake and lysosomal delivery of galactocerebrosidase tagged with the HIV Tat protein transduction domain. *J Neurochem* 104(4):1055-1064.
359. Enquist IB, *et al.* (2009) Successful low-risk hematopoietic cell therapy in a mouse model of type 1 Gaucher disease. *Stem Cells* 27(3):744-752.
360. Storer F, Sargent JD, & Hobbs JR (1987) Regression of the radiological changes of Gaucher's disease following bone marrow transplantation. *Br J Radiol* 60(720):1189-1195.
361. Tsai P, *et al.* (1992) Allogenic bone marrow transplantation in severe Gaucher disease. *Pediatr Res* 31(5):503-507.
362. Filipovich AH, *et al.* (2005) National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. *Biol Blood Marrow Transplant* 11(12):945-956.
363. Correll PH, Colilla S, Dave HP, & Karlsson S (1992) High levels of human glucocerebrosidase activity in macrophages of long-term reconstituted mice after retroviral infection of hematopoietic stem cells. *Blood* 80(2):331-336.
364. Migita M, *et al.* (1995) Selection of transduced CD34+ progenitors and enzymatic correction of cells from Gaucher patients, with bicistronic vectors. *Proc Natl Acad Sci U S A* 92(26):12075-12079.
365. Nolte JA, Yu XJ, Bahner I, & Kohn DB (1992) Retroviral-mediated transfer of the human glucocerebrosidase gene into cultured Gaucher bone marrow. *J Clin Invest* 90(2):342-348.

366. Kim EY, *et al.* (2005) Long-term expression of the human glucocerebrosidase gene in vivo after transplantation of bone-marrow-derived cells transformed with a lentivirus vector. *J Gene Med* 7(7):878-887.
367. Jin GS, Zhu GD, Zhao ZG, & Liu FS (2012) VP22 enhances the expression of glucocerebrosidase in human Gaucher II fibroblast cells mediated by lentiviral vectors. *Clin Exp Med* 12(3):135-143.
368. McEachern KA, *et al.* (2006) AAV8-mediated expression of glucocerebrosidase ameliorates the storage pathology in the visceral organs of a mouse model of Gaucher disease. *J Gene Med* 8(6):719-729.
369. Hong YB, Kim EY, Yoo HW, & Jung SC (2004) Feasibility of gene therapy in Gaucher disease using an adeno-associated virus vector. *J Hum Genet* 49(10):536-543.
370. Sorge J, Kuhl W, West C, & Beutler E (1987) Complete correction of the enzymatic defect of type I Gaucher disease fibroblasts by retroviral-mediated gene transfer. *Proc Natl Acad Sci USA* 84(4):906-909.
371. Bansal V, *et al.* (1994) Transduction, expression, and secretion of human glucocerebrosidase by murine myoblasts. *Ann N Y Acad Sci* 716:307-320.
372. Ohashi T, *et al.* (1992) Efficient transfer and sustained high expression of the human glucocerebrosidase gene in mice and their functional macrophages following transplantation of bone marrow transduced by a retroviral vector. *Proc Nat Acad Sci USA* 89(23):11332-11336.
373. Dunbar CE, *et al.* (1998) Retroviral transfer of the glucocerebrosidase gene into CD34+ cells from patients with Gaucher disease: in vivo detection of transduced cells without myeloablation. *Hum Gene Ther* 9(17):2629-2640.
374. Way KJ, *et al.* (2009) The generation and properties of human macrophage populations from hemopoietic stem cells. *J Leukoc Biol* 85(5):766-778.
375. Chu K, Conetta KG, & Econs MJ (2008) Efficient and stable gene expression into human osteoclasts using an HIV-1-based lentiviral vector. *DNA Cell Biol*. 27(6):315-320.
376. Ramnaraine ML, Mathews WE, & Clohisy DR (2012) Lentivirus transduction of human osteoclast precursor cells and differentiation into functional osteoclasts. *Bone* 50(1):97-103.
377. Moscatelli I, *et al.* (2013) Lentiviral gene transfer of TCIRG1 into peripheral blood CD34(+) cells restores osteoclast function in infantile malignant osteopetrosis. *Bone* 57(1):1-9.
378. Ponce E, Witte DP, Hung A, & Grabowski GA (2001) Temporal and spatial expression of murine acid beta-glucosidase mRNA. *Mol Genet Metab* 74(4):426-434.
379. Doll RF & Smith FI (1993) Regulation of expression of the gene encoding human acid beta-glucosidase in different cell types. *Gene* 127(2):255-260.
380. Doll RF, Bruce A, & Smith FI (1995) Regulation of the human acid beta-glucosidase promoter in multiple cell types. *Biochim Biophys Acta* 1261(1):57-67.
381. Booth C, Gaspar HB, & Thrasher AJ (2011) Gene therapy for primary immunodeficiency. *Curr Opin Pediatr* 23(6):659-666.
382. Ghattas IR, Sanes JR, & Majors JE (1991) The encephalomyocarditis virus internal ribosome entry site allows efficient coexpression of two genes from a recombinant provirus in cultured cells and in embryos. *Mol Cell Biol* 11(12):5848-5859.
383. Baens M, *et al.* (2006) The dark side of EGFP: defective polyubiquitination. *PLoS One* 1:e54.

384. van Es HH, Veldwijk M, Havenga M, & Valerio D (1997) A flow cytometric assay for lysosomal glucocerebrosidase. *Analytical Biochemistry* 247(2):268-271.
385. Wenger DA, Clark C, Sattler M, & Wharton C (1978) Synthetic substrate beta-glucosidase activity in leukocytes: a reproducible method for the identification of patients and carriers of Gaucher's disease. *Clin Genet* 13(2):145-153.
386. Chamoles NA, Blanco M, Gaggioli D, & Casentini C (2002) Gaucher and Niemann-Pick diseases - enzymatic diagnosis in dried blood spots on filter paper: retrospective diagnoses in newborn-screening cards. *Clin Chim Acta* 317(1-2):191-197.
387. Morishima S, *et al.* (2003) Expression and role of mannose receptor/terminal high-mannose type oligosaccharide on osteoclast precursors during osteoclast formation. *J Endocrinol* 176(2):285-292.
388. Kim EY, *et al.* (2004) Expression and secretion of human glucocerebrosidase mediated by recombinant lentivirus vectors in vitro and in vivo: implications for gene therapy of Gaucher disease. *Biochem Biophys Res Commun* 318(2):381-390.
389. Aerts JM, *et al.* (1987) Relationship between the two immunologically distinguishable forms of glucocerebrosidase in tissue extracts. *Eur J Biochem* 163(3):583-589.
390. Ciana G, Deroma L, Franzil AM, Dardis A, & Bembi B (2012) Long-term bone mineral density response to enzyme replacement therapy in a retrospective pediatric cohort of Gaucher patients. *J Inherit Metab Dis* 35(6):1101-1106.
391. Gentner B, *et al.* (2010) Identification of hematopoietic stem cell-specific miRNAs enables gene therapy of globoid cell leukodystrophy. *Sci Transl Med* 2(58):58ra84.
392. Nathwani AC, *et al.* (2011) Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med* 365(25):2357-2365.
393. Palfi S, *et al.* (2014) Long-term safety and tolerability of ProSavin, a lentiviral vector-based gene therapy for Parkinson's disease: a dose escalation, open-label, phase 1/2 trial. *Lancet* 383(9923):1138-1146.
394. Bennett J, *et al.* (2012) AAV2 gene therapy readministration in three adults with congenital blindness. *Sci Transl Med* 4(120):120ra115.
395. Yla-Herttuala S (2012) Endgame: glybera finally recommended for approval as the first gene therapy drug in the European union. *Mol Ther*, (United States), Vol 20, pp 1831-1832.
396. Gaudet D, *et al.* (2013) Efficacy and long-term safety of alipogene tiparvovec (AAV1-LPLS447X) gene therapy for lipoprotein lipase deficiency: an open-label trial. *Gene Ther* 20(4):361-369.
397. Biffi A, *et al.* (2011) Lentiviral vector common integration sites in preclinical models and a clinical trial reflect a benign integration bias and not oncogenic selection. *Blood*, (United States), Vol 117, pp 5332-5339.
398. Montini E, *et al.* (2006) Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nat Biotechnol* 24(6):687-696.
399. Kustikova OS, *et al.* (2009) Cell-intrinsic and vector-related properties cooperate to determine the incidence and consequences of insertional mutagenesis. *Mol Ther* 17(9):1537-1547.
400. Lewis PF & Emerman M (1994) Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J Virol* 68(1):510-516.

401. Miller DG, Adam MA, & Miller AD (1990) Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol Cell Biol* 10(8):4239-4242.
402. Katz RA, Greger JG, Boimel P, & Skalka AM (2003) Human immunodeficiency virus type 1 DNA nuclear import and integration are mitosis independent in cycling cells. *J Virol* 77(24):13412-13417.
403. Cartier N, *et al.* (2009) Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science* 326(5954):818-823.
404. Shapiro E, *et al.* (2000) Long-term effect of bone-marrow transplantation for childhood-onset cerebral X-linked adrenoleukodystrophy. *Lancet*, (England), Vol 356, pp 713-718.
405. Capotondo A, *et al.* (2012) Brain conditioning is instrumental for successful microglia reconstitution following hematopoietic stem cell transplantation. *Proc Natl Acad Sci U S A* 109(37):15018-15023.
406. Biffi A, *et al.* (2013) Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. *Science*, (United States), Vol 341, p 1233158.
407. Chen RL, Hou JW, Chang PY, Tsai FJ, & Wang PJ (2007) Matched unrelated bone marrow transplantation without splenectomy for a child with Gaucher disease caused by homozygosity of the L444P mutation, who also suffered from schizencephaly. *J Pediatr Hematol Oncol*, (United States), Vol 29, pp 57-59.
408. Gupta N, Oppenheim IM, Kauvar EF, Tayebi N, & Sidransky E (2011) Type 2 Gaucher disease: Phenotypic variation and genotypic heterogeneity. *Blood Cells Mol. Dis.* 46(1):75-84.
409. Machaczka M (2013) Allogeneic hematopoietic stem cell transplantation for treatment of Gaucher disease. *Pediatr Hematol Oncol* 30(5):459-461.
410. Stetson DB & Medzhitov R (2006) Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. *Immunity*, (United States), Vol 24, pp 93-103.
411. Stenglein MD, Burns MB, Li M, Lengyel J, & Harris RS (2010) APOBEC3 proteins mediate the clearance of foreign DNA from human cells. *Nat Struct Mol Biol* 17(2):222-229.
412. Kang EM, *et al.* (2010) Retrovirus gene therapy for X-linked chronic granulomatous disease can achieve stable long-term correction of oxidase activity in peripheral blood neutrophils. *Blood* 115(4):783-791.
413. Gaspar HB, *et al.* (2006) Successful reconstitution of immunity in ADA-SCID by stem cell gene therapy following cessation of PEG-ADA and use of mild preconditioning. *Mol Ther* 14(4):505-513.
414. Gaspar HB, *et al.* (2009) How I treat ADA deficiency. *Blood* 114(17):3524-3532.
415. Varma N, Janic B, Ali M, Iskander A, & Arbab A (2011) Lentiviral Based Gene Transduction and Promoter Studies in Human Hematopoietic Stem Cells (hHSCs). *J Stem Cells Regen Med* 7(1):41-53.
416. Ramezani A, Hawley TS, & Hawley RG (2000) Lentiviral vectors for enhanced gene expression in human hematopoietic cells. *Mol Ther* 2(5):458-469.
417. He W, *et al.* (2006) Development of a synthetic promoter for macrophage gene therapy. *Hum Gene Ther* 17(9):949-959.
418. Levin MC, *et al.* (2012) Evaluation of macrophage-specific promoters using lentiviral delivery in mice. *Gene Ther* 19(11):1041-1047.
419. Visigalli I, *et al.* (2010) The galactocerebrosidase enzyme contributes to the maintenance of a functional hematopoietic stem cell niche. *Blood* 116(11):1857-1866.

420. Liu Y, *et al.* (1998) Mice with type 2 and 3 Gaucher disease point mutations generated by a single insertion mutagenesis procedure. *Proc Natl Acad Sci U S A* 95(5):2503-2508.
421. Xia H, Mao Q, & Davidson BL (2001) The HIV Tat protein transduction domain improves the biodistribution of beta-glucuronidase expressed from recombinant viral vectors. *Nat Biotechnol* 19(7):640-644.
422. Pelled D, Shogomori H, & Futerman AH (2000) The increased sensitivity of neurons with elevated glucocerebroside to neurotoxic agents can be reversed by imiglucerase. *J Inherit Metab Dis* 23(2):175-184.
423. van Gelder CM, *et al.* (2014) Enzyme therapy and immune response in relation to CRIM status: the Dutch experience in classic infantile Pompe disease. *J Inherit Metab Dis*.
424. Banugaria SG, *et al.* (2012) Persistence of high sustained antibodies to enzyme replacement therapy despite extensive immunomodulatory therapy in an infant with Pompe disease: need for agents to target antibody-secreting plasma cells. *Mol Genet Metab* 105(4):677-680.
425. Markovitz RC, Healey JF, Parker ET, Meeks SL, & Lollar P (2013) The diversity of the immune response to the A2 domain of human factor VIII. *Blood* 121(14):2785-2795.
426. Ragni MV, *et al.* (2009) Risk factors for inhibitor formation in haemophilia: a prevalent case-control study. *Haemophilia* 15(5):1074-1082.
427. Linthorst GE, Hollak CE, Donker-Koopman WE, Strijland A, & Aerts JM (2004) Enzyme therapy for Fabry disease: neutralizing antibodies toward agalsidase alpha and beta. *Kidney Int, United States*, Vol 66, pp 1589-1595.
428. Vedder AC, *et al.* (2008) Treatment of Fabry disease with different dosing regimens of agalsidase: effects on antibody formation and GL-3. *Mol Genet Metab, United States*, Vol 94, pp 319-325.
429. Pittenger MF, *et al.* (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* 284(5411):143-147.
430. Kuznetsov SA, *et al.* (1997) Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. *J Bone Miner Res* 12(9):1335-1347.